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NEW APPROACHES FOR VOLTAMMETRIC DETERMINATION OF TUMOUR BIOMARKERS AND ANTIDOTES IN URINE

Nové přístupy pro voltametrické stanovení tumorových biomarkerů a antidot v moči

Ph.D. Thesis

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Prague, 10th August 2020 ……..……

Mgr. Vojtěch Hrdlička

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Abstract

This Ph. D. thesis presents new methods for the determination of selected clinically relevant electrochemically active compounds.

The first part deals with development of determination of tumour biomarkers homovanillic acid (HVA) and vanillylmandelic acid (VMA) in human urine with the use of hollow-fibre based liquid-phase microextraction (HF-LPME) and differential pulse voltammetry (DPV) at cathodically pre-treated boron doped diamond electrode (BDDE). Optimum conditions for HF-LPME-DPV of HVA and VMA were as follows: butyl benzoate as supported liquid membrane formed on porous polypropylene hollow-fibre, 0.1 mol L^{-1} HCl as donor phase and 30 min extraction time. Optimum acceptor phases were 0.1 mol L^{-1} phosphate buffer of pH 6 with ionic strength set to 0.55 mol L^{-1} for HVA and 0.1 mol L^{-1} NaOH for VMA, respectively. HF-LPME-DPV concentration dependencies for HVA and VMA were linear in the range from 0.4 to 100 μmol L^{-1} and 0.5 to 100 μmol L^{-1}. Limits of quantification (*LOQ*)/detection (*LOD*) were 1.2/0.4 μmol L^{-1} for HVA and 1.7/0.5 μmol L^{-1} for VMA, respectively. The applicability of the developed methods was verified by analysis of human urine.

In the second part, voltammetric behaviour of heavy metal poisoning antidote 2,3-dimercapto-1-propane-sulfonic acid (DMPS) was investigated with the use of various voltammetric techniques at polished (p-AgSAE) and at meniscus modified (m-AgSAE) silver solid amalgam electrode. The electrode process of DMPS involves two consecutive reductive desorptions with coupled proton/electron transfer preceded by a kinetic process, which is prevalent at higher scan rates as revealed by EVLS. Voltammetric and complexation behaviour of DMPS in the presence of Pb^2 was further investigated by voltammetric titrations confirming multiple processes of reductive desorption, complex formation, and transmetalation.

A new method for the determination of DMPS was developed using differential pulse cathodic stripping voltammetry (DPCSV). Optimum conditions for DPCSV were as follows: Britton-Robinson buffer (BRB) of pH 10, E_{acc} = −0.2 V and t_{acc} = 30 s for p-AgSAE and BRB of pH 5, E_{acc} = 0.0 V and t_{acc} = 15 s for m-AgSAE. *LOQ*s and *LOD*s were 0.3 and 0.1 μmol L^{-1} at m-AgSAE and 0.8 and 0.3 μmol L^{-1} at p-AgSAE, respectively. Practical applicability was successfully tested by determination of DMPS in drug Dimaval® and human urine.

Abstrakt

Tato dizertační práce představuje nové metody pro stanovení vybraných klinicky významných elektrochemicky aktivních látek.

První část se zabývá optimalizací stanovení rakovinových biomarkerů kyseliny homovanilové (HVA) a kyseliny vanilmandlové (VMA) v lidské moči s využitím kapalinové mikroextrakce do dutého vlákna (HF-LPME) a diferenční pulzní voltametrie (DPV) na katodicky předupravené borem dopované diamantové elektrodě (BDDE). Optimální podmínky pro HF-LPME-DPV stanovení HVA a VMA byly následující: butylbenzoátová tekutá membrána ukotvená na polypropylenovém dutém vlákně, donorový roztok 0.1 mol L^{-1} HCl a čas extrakce 30 min. Optimální akceptorové fáze byl fosfátový pufr o pH 6 s iontovou silou upravenou na 0.55 mol L^{-1} pro HVA a 0.1 mol L^{-1} NaOH pro VMA. HF-LPME-DPV koncentrační závislosti byly lineární v rozsahu 0.4 až 100 μmol L^{-1} pro HVA a 0.5 až 100 μmol L^{-1} pro VMA. Meze stanovitelnosti (*LOQ*)/detekce (*LOD*) činily 1.2/0.4 μmol L^{-1} pro HVA a 1.7/0.5 μmol L^{-1} for VMA. Praktická využitelnost této metody byla ověřena na vzorcích lidské moči.

Ve druhé části bylo zkoumáno voltametrické chování antidota otravy těžkými kovy, 2,3-dimerkapto-1-propan-sulfonové kyseliny (DMPS) s využitím různých voltametrických technik na leštěné (p-AgSAE) a rtuťovým meniskem modifikované tuhé amalgámové elektrodě (m-AgSAE). Elektrochemická redukce DMPS zahrnovala dvě následné redukce v adsorbovaném stavu se současným přenosem protonu a elektronu, s předřazeným kinetickým dějem při vyšších rychlostech scanu, jak bylo zjištěno pomocí eliminační voltametrie s lineárním scanem (EVLS). Dále byly studovány voltametrické a komplexační vlastnosti DMPS v přítomnosti Pb^{2+}. Pomocí voltametrických titrací byly identifikovány různé procesy včetně redukce v adsorbovaném stavu, tvorby komplexů a transmetalace.

Byly vyvinuty nové metody pro stanovení DMPS pomocí diferenční pulzní katodické rozpouštěcí voltametrie (DPCVS). Optimální podmínky byly následující: Brittonův-Robinsonův pufr (BRB) pH 10, $E_{acc} = -0.2$ V a t_{acc} = 30 s pro p-AgSAE a BRB pH 5, $E_{acc} = 0.0$ V a t_{acc} = 15 s pro m-AgSAE. Dosažené *LOQ* and *LOD* činily 0.3/0.1 μmol L^{-1} na m-AgSAE a 0.8/0.3 μmol L^{-1} na p-AgSAE. Praktická využitelnost metody byla ověřena na stanovení DMPS v léčivě Dimaval® a v lidské moči.

Keywords

2,3-Dimercapto-1-propanesulfonic acid
Boron doped diamond electrode
Catecholamine metabolites
Cathodic stripping voltammetry
Differential pulse voltammetry
Elimination voltammetry with linear scan
Hollow fibre based liquid phase microextraction
Homovanillic acid
Neuroblastoma biomarkers
Silver solid amalgam electrode
Vanillylmandelic acid

Klíčová slova

2,3-Dimerkapto-1-propansulfonová kyselina
Borem dopovaná diamantová elektroda
Katecholaminové metabolity
Katodická rozpouštěcí voltametrie
Diferenční pulzní voltametrie
Eliminační voltametrie s lineárním scanem
Kapalinová mikroextrakce s využitím dutého vlákna
Kyselina homovanilová
Biomarkery neuroblastomu
Tuhá stříbrná amalgámová elektroda
Kyselina vanilmandlová

List of Abbreviations

AA	ascorbic acid
BDDE	boron doped diamond electrode
BRB	Britton – Robinson buffer
c	molar concentration (mol L^{-1})
CV	cyclic voltammetry
DCCSV	direct current cathodic stripping voltammetry
DMPS	2,3-dimercapto-1-propane-sulfonic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DPASV	differential pulse anodic stripping voltammetry
DPCSV	differential pulse cathodic stripping voltammetry
DPV	differential pulse voltammetry
E	potential (V)
E_{act}	activation potential (V)
EF	enrichment factor
EVLS	elimination voltammetry with linear scan
HF	hollow fibre
HF-LPME	hollow fibre liquid phase microextraction
HVA	homovanillic acid
I	current (A)
I_p	peak current (A)
LOD	limit of detection (µmol L^{-1})
LOQ	limit of quantification (µmol L^{-1})
m-AgSAE	mercury meniscus modified silver solid amalgam electrode
p-AgSAE	polished silver solid amalgam electrode
PBS	phosphate buffer
pK_a	acid dissociation constant
RSD	relative standard deviation (%)
SLM	supported liquid membrane
t_{act}	activation time (s)
t_{ext}	extraction time (min)
UA	uric acid
v	scan rate (V s^{-1})

V_{AS}	volume of acceptor solution (mL)
V_{DS}	volume of donor solution (mL)
VMA	vanillylmandelic acid

Contents

1. Aims of the work

1. Development of new methods for the determination of urinary homovanillic (HVA) and vanillylmandelic acid (VMA) with the use of hollow-fibre based liquid phase microextraction (HF-LPME) with subsequent voltammetric detection.
    - Modification and miniaturization of existing voltammetric methods for determination of HVA and VMA in 10 μL of basic/neutral HF-LPME extract.
    - Optimization of HF-LPME-DPV of HVA and VMA.
    - Verification of the developed method(s) on urine samples.

2. Development of a new method for determination of 2,3-dimercapto-1-propane-sulfonic acid (DMPS) at silver solid amalgam electrodes.
    - Study of voltammetric behaviour of DMPS and the electrode process with the use various voltammetric methods including elimination voltammetry with linear scan.
    - Optimization of cathodic stripping voltammetry of DMPS.
    - Application of the newly developed method(s) for analysis of real drug and urine samples.

2. Introduction

This thesis presents results acquired during my Ph.D. studies at the Faculty of Science of Charles University and J. Heyrovský Institute of Physical Chemistry of the CAS under framework of UNESCO Laboratory of Environmental Electrochemistry. Majority of the text is based on published or submitted articles, which are attached as appendices. These works will be cited as follows:

[1] **Vojtěch Hrdlička**, Tomáš Navrátil, Jiří Barek: Application of hollow fibre based microextraction for voltammetric determination of vanillylmandelic acid in human urine. Journal of Electroanalytical Chemistry 835, 130-136 (2019).

[2] **Vojtěch Hrdlička**, Marta Choińska, Beatriz Ruiz Redondo, Jiří Barek, Tomáš Navrátil: Determination of heavy metal poisoning antidote 2, 3-dimercapto-1-propanesulfonic acid using silver solid amalgam electrode. Electrochimica Acta 354, 136623. (2020).

[3] **Vojtěch Hrdlička**, Jiří Barek, Tomáš Navrátil: Differential pulse voltammetric determination of homovanillic acid as a tumour biomarker in human urine after hollow fiber-based liquid-phase microextraction. Manuscript submitted for publication (2020).

Since the discovery of polarography by J. Heyrovský, electrochemical methods have been indispensable tools for analytical and physical chemistry[4]. Recent development of new voltammetric methods deals with new challenges such as sensing in complex biological matrices including blood plasma, cerebrospinal fluid or urine [5, 6].

Direct electroanalysis without a separation step is often impossible in these complex matrices, due to presence of multitude of electrochemically active interferences or high-molecular species that can adsorb to the electrode surface. These adsorption and chemisorption processes are generally more prominent at the metallic electrodes than at the carbon ones and could present a major obstacle for the use of voltammetric techniques, especially adsorptive stripping voltammetry [7].

Even carbon and boron doped diamond electrodes (BDDE), which are generally very resistant to fouling are still prone, at least to some extent, to passivation by products of electrode reactions, including oxidation products of phenolic [8] or amino [9] compounds that

generate passivating polymer films.

Moreover, the analysis of urine is even more complicated in the area of positive potentials due to presence of electrochemically oxidizable uric acid (UA) and ascorbic acid (AA) in up to milimolar concentrations. Other electrochemically active interferences are present in lower concentrations, such as catecholamines and their metabolites [10]; or various phenolic compounds excreted by cytochrome P450 pathway [11].

All these factors, among many others need to be considered for the selection of suitable electrode material and sample pre-treatment method for intended purpose.

2.1 Homovanillic acid and Vanillylmandelic acid

Homovanillic acid (HVA) and vanillylmandelic acid (VMA) are final metabolic products of catecholamines dopamine and epinephrine in human body (Fig. 1) [12] and well-established clinical biomarkers of various diseases with altered excretion of catecholamines [10, 13]. Moreover, HVA is used as a target molecule for overall dopamine activity in human body [14].

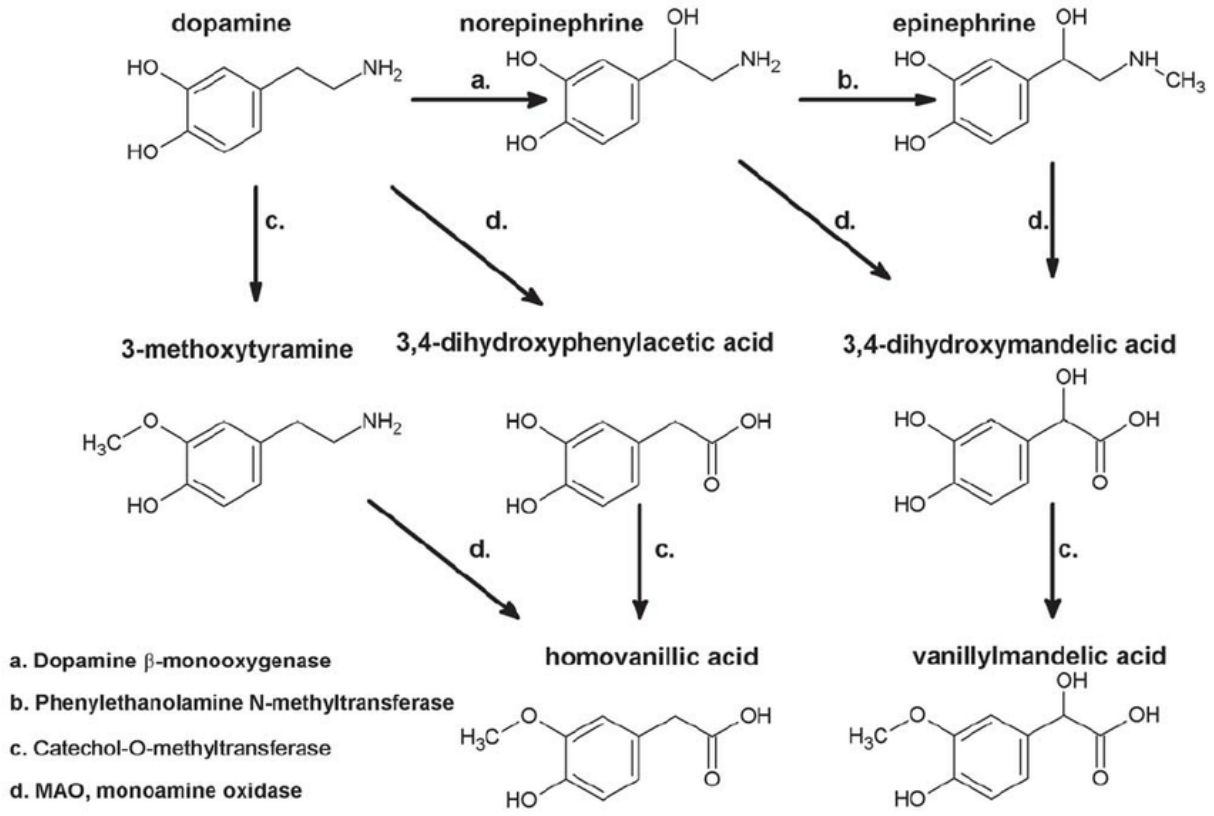

Fig. 1 *Scheme of catecholamine catabolism in human body with final products HVA and VMA [15].*

Elevated levels of HVA and VMA in blood, urine or cerebrospinal fluid are typical for autism [16], bulimia [17], posttraumatic stress disorder [18] and cancerous diseases of

catecholamine producing glands: tumour of adrenal medulla pheochromocytoma [19, 20], tumours of neural crest [21], and neuroblastoma [22, 23]. Lowered levels are linked to depression [24-26], Parkinson´s disease [10] and Wilson´s disease [13, 27]. HVA/VMA ratio is used for diagnosis of Menkes disease [28, 29].

Determination of HVA and VMA in bodily fluids is relevant for diagnosis [30], monitoring of disease progression [31, 32], drug dosage optimization [33] and screening tests [34]. Screening tests for neuroblastoma with the use of HVA and VMA as target molecules reported a significant decrease of the patients mortality [22, 30, 35].

Molar concentration of urinary HVA and VMA in healthy 6-month-old infants is 34.6 ± 7.6 μmol L^{-1} and 17.1 ± 7.9 μmol·L^{-1}, respectively, whilst the cut-off concentration increase for diagnosis neuroblastoma is 2.5 of standard deviation, hence 53.6 μmol and 34.3 μmol L^{-1}, for HVA and VMA, respectively [21, 36-40]. These values are also age-dependent and influenced by dietary flavonoids, which increase excretion of HVA, leading to false positive results [41].

For clinical purposes, levels of HVA and VMA are often expressed as a ratio to creatinine, due to approximately constant excretion of creatinine over time with regard to sex and age and to take correction for different drinking regime [36]. This normalizes the measured HVA or VMA concentration to the dilution of urine. Sampling strategies, including collection of urine over 24 hours are discussed in dedicated publications [19, 42, 43]. Concentrations of HVA and VMA in urine samples stored at 4 and −20 °C are stable for a year [44].

Techniques for HVA and VMA determination include liquid chromatography [45-48], gas chromatography [16, 49-52], thin layer chromatography [53-55], capillary electrophoresis [40, 56], often with a sample preparation step such as centrifugation, liquid/liquid extraction, or solid phase extraction [47], which further increases already substantial acquisition and operational cost of the equipment.

Electrochemical methods, including potentiometry [57, 58], flow-injection analysis [59] and voltammetry[60, 61] present viable alternatives from the standpoint of their simplicity, high-sensitivity, low-cost and often sufficient selectivity as shown in a dedicated review by Němečková-Makrlíková [62]. Due to the presence of electrochemically oxidizable phenolic group, both HVA and VMA can be voltammetrically determined on various carbon electrodes including paste [46], composite [63], plane edge [64], polymer modified [60], screen printed [61], nanoparticle modified electrodes [65] or BDDE [60]. Mechanism of HVA

electrochemical oxidation on carbon electrodes is pH dependent. The process involves transfer of two electrons/one proton at neutral pH or two electrons/two protons at acidic pH [64, 66] (Fig. 2), resulting in one voltammetric peak.

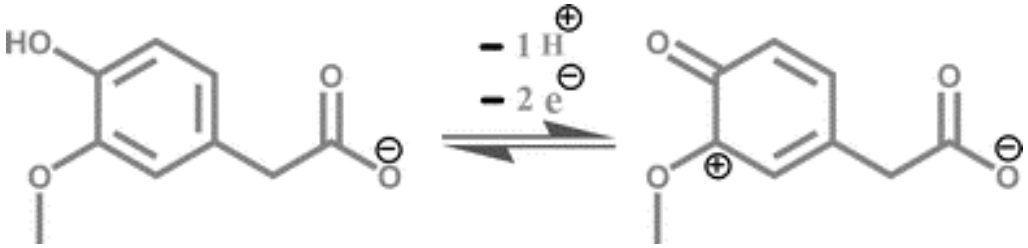

Fig. 2 *Scheme of electrochemical oxidation of HVA at neutral pH.* [66]

Products of the HVA oxidation can include electrochemically active 4-acetoquinone and DOPAC, which can adsorb on the electrode surface and manifest as new distinct peaks in consecutive voltammetric scans, especially at glassy carbon or metallic electrodes [67, 68].

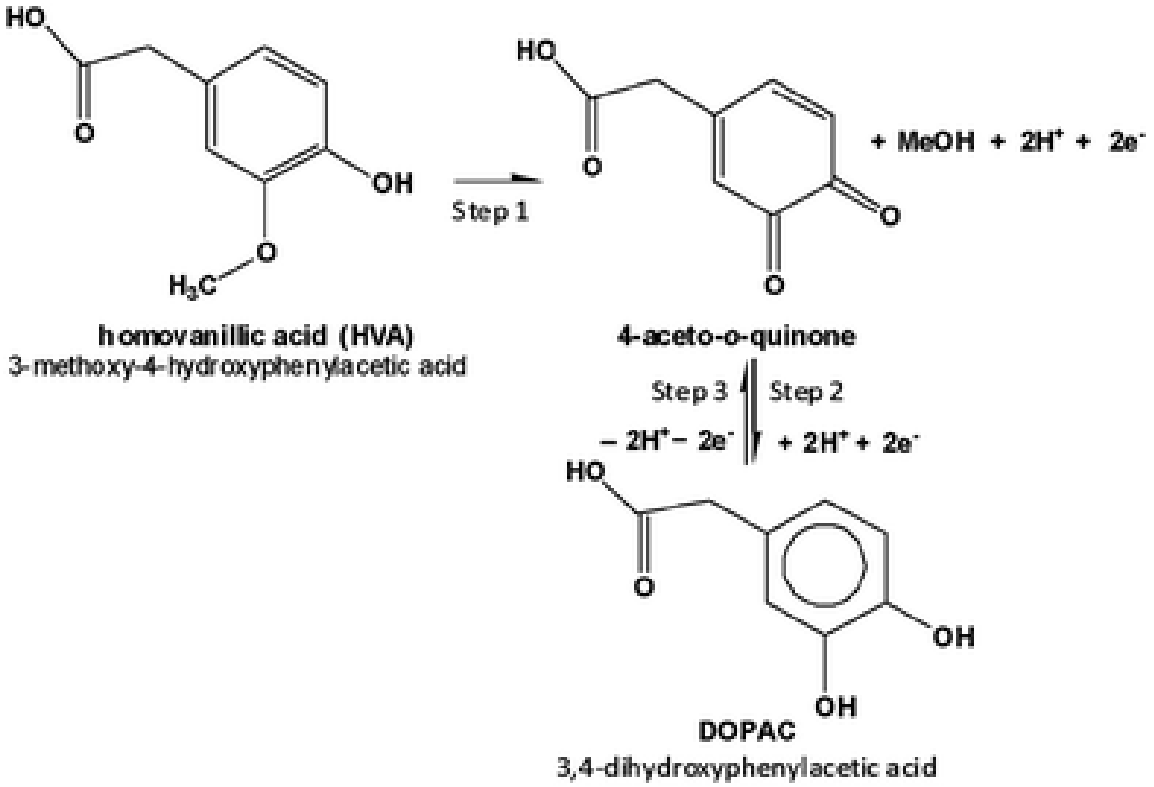

Fig. 3 *Scheme of DOPAC formation during electro-oxidation of HVA.* [68]

VMA oxidation occurs in analogous way to HVA, followed by a second step, distinct only to VMA, consisting of decarboxylation and formation of vanillin [64] or a ring rearrangement

[69] and second two electron/two protons transfer process producing O-quinone (Fig. 4), resulting in two peaks.

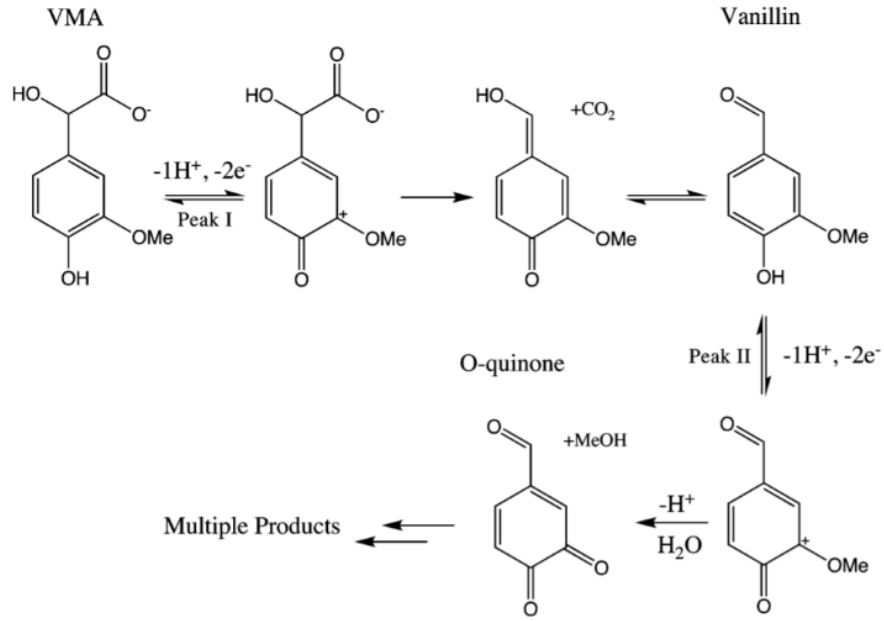

Fig. 4 *Proposed mechanism for the electrochemical oxidation of VMA at plane edge graphite or carbon nanotubes modified carbon electrodes.* [64]

Voltammetric signals of HVA and VMA are separated sufficiently at plane edge graphite or carbon nanotubes modified electrodes [64], or when molecularly imprinted polymers are used [70, 71].

Alternatively, if the first oxidation peaks of HVA and VMA are merged into one and the second peak corresponds only to VMA concentration, HVA concentration can be calculated with the knowledge of VMA concentration and HVA/VMA peak ratios at particular conditions. This case occurs on screen printed [61], carbon paste [46], composite carbon [63] and cathodically pre-treated/H-terminated BDDE. On the other hand anodically pre-treated BDDE is an exemption, as the separation of peaks is not sufficient for a simple HVA/VMA discrimination without more complicated mathematical post-processing [60].

Reported optimum pH for voltammetric determination of HVA and VMA is typically pH 2, 3 or 7 [62]. It is notable that strongly basic conditions are not optimal or completely ineffective for voltammetric determination of HVA or VMA in micromolar levels. This is especially problematic for the use of voltammetry for determination of HVA or VMA in combination with three phase HF-LPME. This is discussed later in following chapter 3.1.1.

The above mentioned voltammetric methods often reach submicromolar limits of

detection and are able to determine both VMA and HVA in a single experiment. However, a direct voltammetric analysis of HVA and VMA in urine is apparently impossible due to the sheer amount of various urinary interferences. An exemption is a recent study by Fu [65] that reports fantastic parameters such as practically 100% recoveries of HVA and VMA in urine samples with very low RSDs < 2.5%, apparently ignoring the fact, that HVA and VMA is already present in urine in concentrations comparable to those of the standard additions, raising questions about practical applicability of the method.

In order to take full advantage of electroanalytical methods, they can be combined with a suitable sample preparation procedure, such as hollow-fibre based liquid microextraction (HF-LPME).

2.2 Hollow fibre based liquid microextraction

HF-LPME is a relatively new method for clean-up of complex samples. Porous disposable plastic hollow fibre (HF) serves as a carrier of supported liquid membrane (SLM) inside the pores of the fibre [72]. SLM separates acceptor phase inside the fibre lumen and donor phase in the bulk solution. Analyte is transported across the SLM by diffusion, pre-concentration is based on partition coefficient of the analyte in the used SLM, donor and acceptor phases [73]. The SLM is formed by dipping of the fibre into a water-immiscible organic solvent, which is held inside the fibre pores by capillary forces [74]. Two phase HF-LPME is basically an improved single drop liquid phase extraction and therefore uses the same solvent for formation of SLM the acceptor solution [75]. This arrangement is usable especially in combination with analytical techniques such as gas chromatography or mass spectrometry [76].

More complicated three phase HF-LPME uses aqueous donor and aqueous acceptor phases with their respective pHs set so that the analyte of interest is in its most soluble form in the acceptor solution, e.g. acidic analyte would be typically extracted from acidic donor solution across SLM into basic acceptor solution (Fig. 5). In such arrangement, the transport of charged basic compounds from the donor solution into SLM is stopped, whilst the neutral compounds can be extracted, but are not pre-concentrated [77, 78].

Selection of solvent for SLM formation and pH of donor and acceptor solutions is critical for the extraction selectivity and efficiency, with regard to the polarity, partition coefficient, and acid-base dissociation constants of the analyte and other components of sample [79]. Viscosity of SLM can be also a factor for extraction efficiency of less mobile analytes.

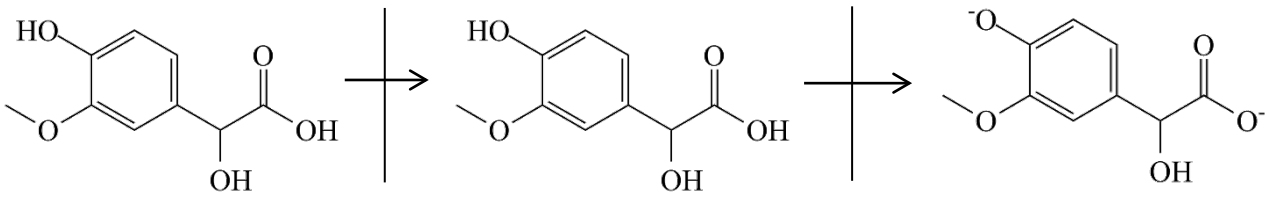

Fig. 5 *Scheme of three-phase liquid/liquid/liquid hollow fibre extraction of VMA* [1].

Enrichment/pre-concentration factors (*EF*) of HF-LPME vary greatly, typically from tens to hundreds [80-82]. Extremely high *EF*s (up to 100 000) have been reported for triazine herbicides [83]. More polar compounds, e.g. ibuprofen [84], clofibric acid [85], salicylic acid [78], aniline derivatives [86], aromatic amines [87], nitrophenols [88], hydrazine or ephedrine [89] typically have much lower *EF*s (the order of tens) after 30-60 min of extraction. This is caused by lower partition coefficient between a polar compound and SLM, which needs to retain its hydrophobicity to be stable. Suitable SLMs for HF-LPME of more polar compounds include aliphatic alcohols, especially 1-octanole, dihexyl ether, toluene, dodecane or esters of benzoic acid [90, 91].

Although the three-phase HF-LPME is most commonly used with liquid chromatography or capillary electrophoresis, two studies dealt with its combination with voltammetry for determination of Hg^{2+} in rice [92] and desipramine in urine [90]. Voltammetric detection is more common in combination with other methods that use hollow fibres, namely electromembrane extraction [93-99] or hollow fibre liquid/solid extraction [81, 100-102].

The aqueous acceptor solution of three phase HF-LPME can be transferred at screen printed electrode surface for voltammetric analysis without any further treatment [97] or the voltammetric experiment can be performed *in-situ* inside the fibre with the use of a miniaturized electrode [90]. For a voltammetric analysis after HF-LPME, SLM solvent choice is obviously limited to electrochemically inactive solvents with respect to the used working electrode material, pH and potential range, particularly if the SLM is sparingly soluble in the acceptor solution. The purity of SLM solvent from the standpoint of electrochemically active compounds, environmental impact, cost and toxicity are other factors to be considered.

HF-LPME is considered to be greener and more environmentally friendly alternative to liquid phase microextraction, due to negligible consumption of solvents [85]. Some of the

most recent trends in HF-LPME are 3D printed platforms for automation of sample pre-treatment [103], electromembrane extraction [104], and the use of sheet porous liquid membranes with micro-titration plates (PALME) for simultaneous extraction of large amount of samples at once [74, 105].

2.3 2,3-Dimercapto-1-propanesulfonic acid

DMPS (2,3-dimercapto-1-propanesulfonic acid, Fig. 6) is a synthetic dithiol antidote for heavy metal poisoning [106-108]. The abbreviation DMPS will be used, when appropriate, for both dithiol sulfonic acid, its sodium salt and the corresponding dissociation states ($pK_{a\ (sulfonic)}$ = 1.1; $pK_{a\ 2,3\ (thiols)}$ = 9.4, 12.1) [109].

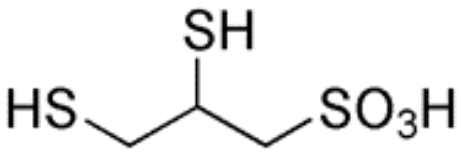

Fig. 6 *Structure of DMPS*

DMPS is used for chronic and acute intoxications by various heavy metals including mercury, cadmium, bismuth, arsenic, and lead [106, 110-113]. DMPS can be also utilized for treatment of Wilson´s disease [114, 115], diabetic polyneuropathy [116], amyloidosis [117], and as an antidote for hemotoxic snakebite [118]. DMPS in a form of sodium salt is an active ingredient of commercially available drugs Dimaval, Unithiol and Mercuval [106].

Determination of DMPS is important for dosage optimization and monitoring of treatment [113]. Electroanalytical techniques, in particular voltammetry, have high potential of applicability for this purpose due to their sensitivity, speed and simplicity [5, 119]. Besides the analytical use, voltammetric techniques can be employed for the study of electrode mechanism and complexing behaviour of thiolate antidotes as well [120-123].

Only one published study has dealt with voltammetric determination of DMPS with reported *LOQ* of 41 μmol L^{-1} at multiwall carbon nanotube modified glassy carbon electrode [124]. Presumably, the sensitivity could have been improved substantially with the use of stripping voltammetry at a silver solid amalgam electrode (AgSAE) [125-127]. Another reason for the selection of AgSAE was significant difference of electrochemically oxidizable and reducible interferences in urine. Whilst the voltammetric analysis of trace components in urine in the area of positive potentials is often extremely difficult, the use of AgSAEs in the area of negative potentials is well documented and less complicated [128-133]. AgSAEs have become a proven alternative to mercury electrodes with similar properties including high sensitivity and wide negative potential window in addition to their mechanical stability and negligible toxicity [134-137].

Thiols oxidatively chemisorb on mercury [138, 139] and silver [140, 141] surfaces,

readily forming semi-covalent metal-thiol bonds, even at open-cell potential [126, 142]. The deposition can be controlled by application of external potential, as an accumulation step for cathodic stripping voltammetry (Eq. 1)[125, 127, 130, 131].

$$\mathrm{Hg + R\text{-}SH} \underset{\textit{chemisorption}}{\overset{\textit{cathodic stripping}}{\rightleftharpoons}} \mathrm{Hg\text{-}S\text{-}R + e^- + H^+} \quad \textbf{(Eq. 1)}$$

The reverse reaction is typically a reduction in adsorbed state, eventually followed by subsequent desorption of the thiol, with regard to the solubility of the thiolate compound [143]. Reduction potential of a chemisorbed thiolate depends strongly on pH (proton coupled reaction), used working electrode [142] and overall hydrophobicity of the molecule. Reduction potentials of bulkier aliphatic thiols such as 1-decanethiol are typically shifted towards more negative potentials [143, 144].

Cleaning of AgSAEs is possible by various methods [137, 145] including the application of negative potentials causing hydrogen evolution, repeated potential pulses, cyclic voltammetry scans, and mechanical and/or chemical cleaning. Concerning thiols, a simple cleaning method with the use of cyclic voltammetry was successfully employed even for sparingly soluble undecanethiol [126].

2.4 Elimination voltammetry with linear scan

Elimination voltammetry with linear scan (EVLS) is a mathematical procedure for the study of electrode mechanism [146-148]. EVLS can provide discrimination of voltammetric currents [146], identification of electrode reaction rate determining step [149], separation of voltammetric signal from background at the end of the potential window [147] or even simple estimation of electrode roughness [150]. The major advantage is its simplicity, as the input experimental data are analogous to those used for I vs. v or log/log analysis. Minimum of three DC/LSV/CV experiments is needed, as explained later.

According to the EVLS theory [146, 148, 151], total recorded voltammetric current (I) can be expressed as a sum of partial currents (I_j) (Eq. 2): kinetic current (I_k), charging current (I_c), diffusion current (I_d) and the irreversible current (I_{ir}).

$$I = \sum I_j = I_k + I_c + I_d + I_{ir} \qquad \text{(Eq. 2)}$$

It is also assumed that each partial current I_j can be expressed as a function of scan rate and a function of potential as given in (Eq. 3).

$$I_j = W_j(v)\, Y_j(E) = v^x Y_j(E) \qquad \text{(Eq. 3)}$$

I_j represents selected partial current, $W_j(v)$ represents a function of the scan rate, and $Y_j(E)$ is a function of the potential. $Y_j(E)$ is assumed to be specific for each partial current and independent of scan rate. The elimination function is typically found with the use of at three or more currents: a reference current I at certain scan rate and two other at the half and twice of the reference current scan rate. EVLS was employed for the study of electrode process of HVA and VMA at BDDE, as well as DMPS at m-AgSAE and p-AgSAE.

3. Results and discussion

3.1 HVA and VMA

This chapter presents summary of publications **[1]** and **[3]** with expanded discussion of obtained results, comparison of HVA/VMA HF-LPME-DPV behaviour and provides EVLS of HVA and VMA at cathodically pre-treated BDDE.

3.1.1 Voltammetric determination of HVA and VMA for HF-LPME

Boron doped diamond electrode was chosen as working electrode due to its excellent properties including low background current, wide potential window, high sensitivity and resistance against fouling [5, 152-154]. A method for voltammetric determination of VMA and HVA at anodically pre-treated BDDE was already published by Baluchova [60] with reported optimum pH 3. However, both VMA and HVA are present as anions in neutral or alkaline hollow-fibre extract solution. This causes repulsion between these anions and negatively charged COO^- and OH^- groups on O-terminated BDDE surface; and subsequent shift of HVA and VMA voltammetric peaks to the area of more positive potentials, peak broadening and poor repeatability.

Mild cathodical pre-treatment of BDDE at −1.0 V vs. Ag|AgCl|3M KCl for 15 s was therefore used to solve this problem for both HVA and VMA. Pre-treatment potentials lower than −1.0 V caused gradually increasing background and lower sensitivity and repeatability. End potential of voltammetric scans was set to +0.85 V in order to avoid undesirable oxidation of BDDE surface resulting in O-termination. H-termination can be recovered by application of highly negative potentials (−3.0 V for 10 min) under acidic conditions [155]. Reversing the BDDE termination from O- to H- is generally more difficult than vice versa.

At pH 13, HVA provided a single well-developed peak at +0.40 V, VMA provided two peaks at +0.45 V and +0.68 V. Second and most prominent VMA peak at +0.68 V was used for all further experiments (Fig. 7,8). It is worth noting that most common electrochemically oxidizable interferences UA and AA are oxidized in a peak at +0.2 V, separated from HVA and VMA peaks [1] and do not affect determination of HVA and VMA even at concentrations as high as 1000 μmol L^{-1}.

The dependence of the HVA and VMA peak height on the scan rate was investigated using CV and EVLS with applied scan rates from 0.01 to 1.28 V s^{-1}. The peak currents were all directly proportional to the square root of the scan rate, confirming diffusion-controlled

process. No significant HVA or VMA counter peak appeared, indicating negligible adsorption of DOPAC on BDDE in comparison with glassy carbon electrode [67, 68].

Moreover, the corresponding EVLS curves were calculated according to the theory presented in the introduction chapter. A set of scan rates from 0.01 to 0.08 V s^{-1} was used for both studied compounds. The controlling process of the single HVA oxidation peak at +0.4 V was diffusion with negligible contribution of other partial currents (Fig. 7). Similarly, the first VMA oxidation peak at +0.45 was controlled predominantly by diffusion. The second VMA oxidation peak was controlled by a diffusion process preceded by a kinetic step. This is with agreement to the VMA oxidation mechanisms at carbon electrodes [64, 69].

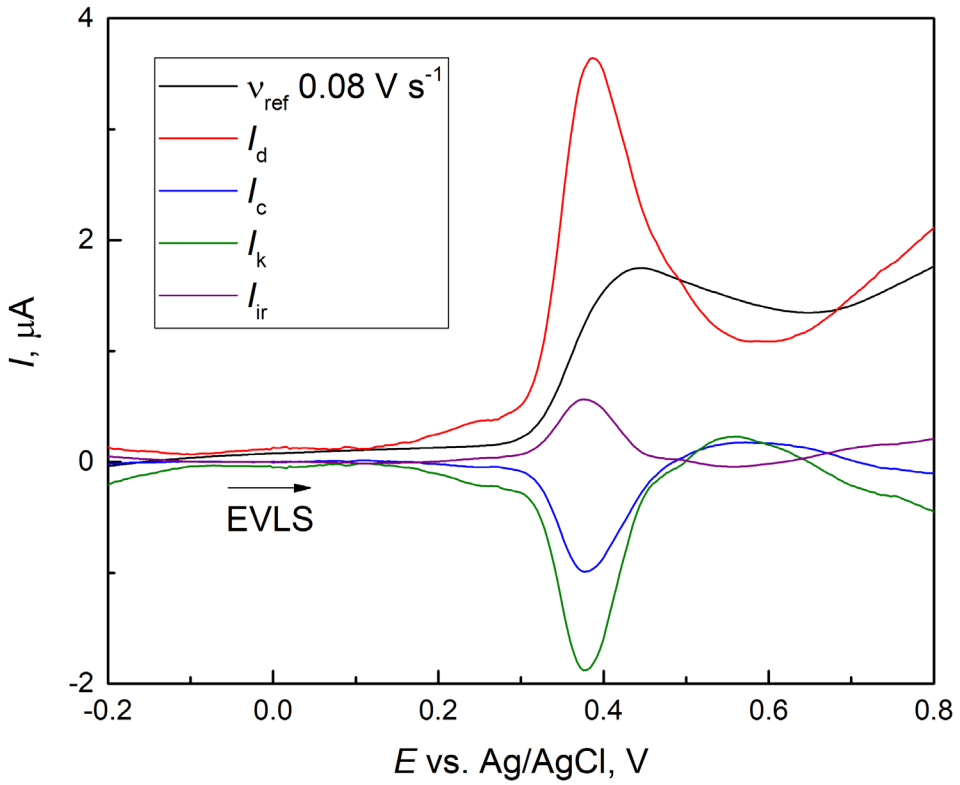

Fig. 7 *EVLS recordings of 0.1 mmol L^{-1} HVA in 0.1 mol L^{-1} NaOH at BDDE. BDDE pretreated at E_{acc} = −1.0 V, t_{acc} = 15 s. Reference scan rate 0.08 V s^{-1}. Elimination curves correspond to diffusion (I_d), charging (I_c), kinetic (I_k), and irreversible (I_{ir}) currents.*

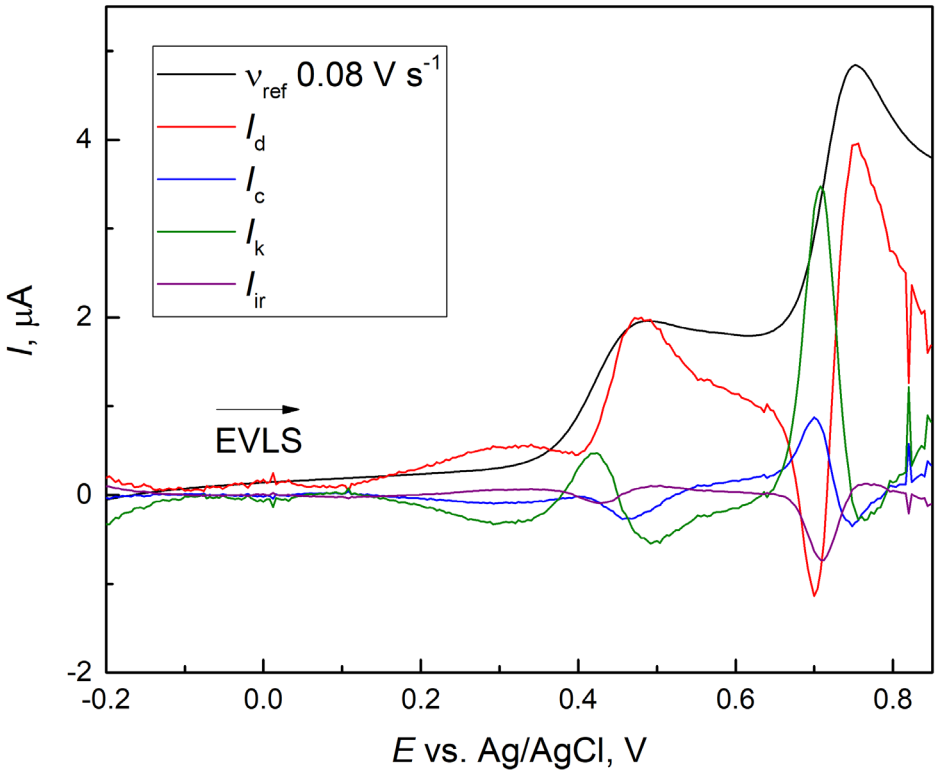

Fig. 8 *EVLS recordings of 0.1 mmol L^{-1} VMA in 0.1 mol L^{-1} NaOH at BDDE. BDDE pre-treated at E_{acc} = −1.0 V, t_{acc} = 15 s. Reference scan rate 0.08 V s^{-1}. Elimination curves correspond to diffusion (I_d), charging (I_c), kinetic (I_k), and irreversible (I_{ir}) currents.*

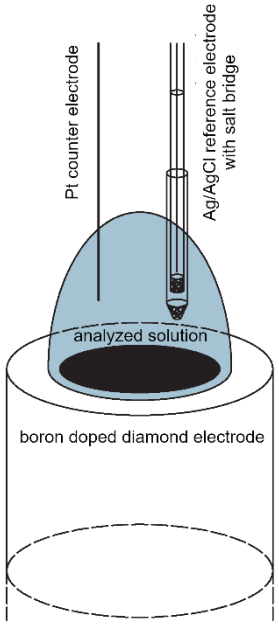

Fig. 9 *Scheme of the used three-electrode system for micro-volume voltammetry.* [3]

Miniaturized three-electrode system with working electrode oriented "*face-up*" for the analysis of 10 μL of sample [3], corresponding to the volume of HF-LPME extract (Fig. 9) was used. Ag|AgCl|3M KCl reference electrode was inserted into a tube with a small frit at the end. The use of the frit was found to be more reliable than a simple Luggin capillary, particularly for analysis of HF-LPME extracts with altered surface tension of the analysed solution. The salt-bridge tube was filled with same solution as HF-LPME acceptor phase (typically 0.1 mol L^{-1} NaOH) in order to avoid undesirable liquid junction potentials or change of pH in the analysed solution. Repeatability of recorded HVA and VMA peak currents at 100 μmol L^{-1} using the miniaturized arrangement was RSD < 6 % (n = 10), which is slightly higher than with the use of conventional three electrode system with 10 mL of solution and RSD < 2%. This demonstrates good overall repeatability of the method with the somewhat higher error caused mainly by very low volume of the sample in the miniaturized arrangement.

DPV calibration curves were constructed in the range from 1-100 and 0.8-100 μmol L^{-1} for VMA and HVA, respectively. *LOQ*s were 2.4 for HVA and 3.3 for VMA. Figures of merit are presented in Table 1. *LOQ*s and *LOD*s of VMA are typically lower than those of HVA, in agreement with slopes of the calibration dependencies. The small difference of *LOQs* and *LODs* in this work is apparently caused by the use of potentiostats with different signal to noise ratio, EcoTribo was used for VMA, whilst PalmSens4 was used for HVA. Same DPV scan rate 0.02 V s^{-1}, pulse potential 0.05 V, time of pulse 100 ms and sampling time 20 ms at the end of the pulse were set at both instruments.

Reached *LOQ*s/*LOD*s at cathodically pre-treated BDDE are more than sufficient for the intended purpose of screening for elevated HVA and VMA in urine. However, direct analysis of urine is impossible, because the HVA/VMA signal is completely overlapped by other electrochemically oxidizable compounds in urine even with the addition of HVA/VMA to the total concentrations as high as 500 μmol^{-1}. The use of sample pre-treatment is therefore necessary.

Table 1

Figures of merits of DPV determination of HVA and VMA in 0.1 mol L^{-1} NaOH at BDDE. DPV scan rate 0.02 V s^{-1}. The coverage intervals were calculated on the significance level $\alpha = 0.05$.

Analyte	**Linear range (µmol L^{-1})**	**Slope (nA µmol^{-1} L)**	**Intercept (nA)**	**Correlation coefficient**	***LOQ* (µmol L^{-1})**	***LOD* (µmol L^{-1})**
HVA	0.8-100	4.620 ± 0.021	6.6 ± 1.1	0.9998	2.4	0.8
VMA	10-100	19.93 ± 0.16	24 ± 10	0.9996		
	1.0-10	24.27 ± 0.64	28.6 ± 4.1	0.9971	3.3	1.0

***** *Insignificant on the significance level $\alpha = 0.05$.*

3.1.2 HF-LPME of HVA and VMA

HF-LPME was carried out with the use of lab-made extraction cell with a stirring bar (Fig. 10). Compared to other published experimental arrangements with open syringes or tubes at the end of the fibre [156-158], tightly sealed hollow fibre was a necessity in order to obtain acceptable repeatability, due to very small volume of the acceptor phase inside the fibre and problems with evaporation of the acceptor phase.

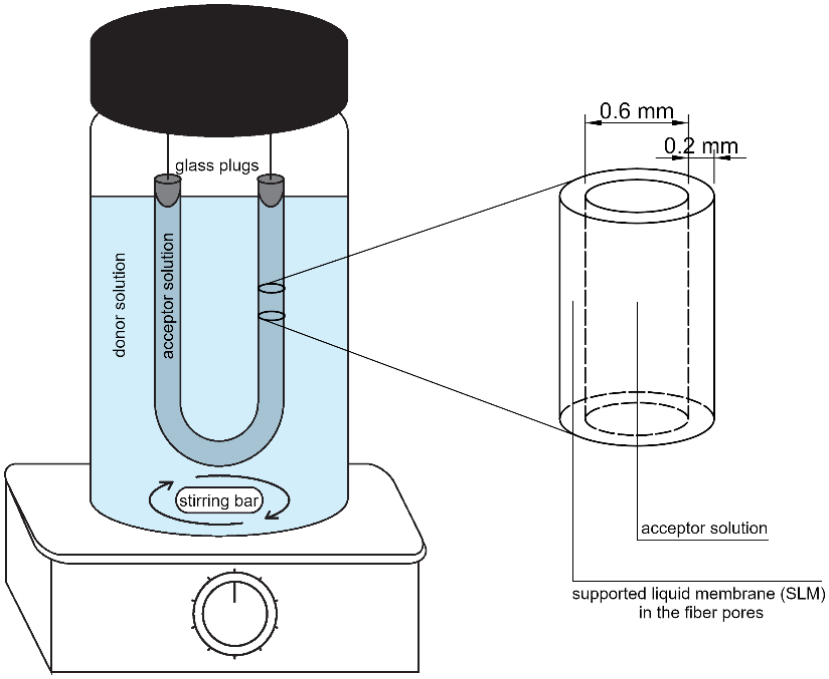

Fig. 10 *Experimental arrangement of the used HF-LPME extraction cell.* [3]

The first step of method development was selection of suitable SLM. This part of optimization is essential, because properties of SLM greatly affect selectivity, extraction efficiency, and repeatability of HF-LPME. Due to high polarity of HVA and VMA, solvent for SLM creation needed to be sufficiently polar in order to allow these compounds to be transported through the membrane to the acceptor solution. This is in contradiction to other requirements on the SLM properties, particularly its immiscibility with water. Although 1-octanol and hexyl benzoate are by far the most used SLM solvents for HF-LPME in general [81, 91], stability of such SLMs is not very good, as they are gradually washed away from the fibre to the donor solution. Surprisingly, there is a study that successfully uses 1-octanol as SLM for HF-LPME whilst shaking, instead of stirring [159]. Butyl benzoate was found to be the optimum SLM, with highest recorded *EF*s 4.67 ± 0.89 and 5.60 ± 0.45 for HVA and

VMA, respectively. Compared to other published HF-LPME methods, obtained *EF*s are rather low, this is supposedly caused by high polarity of HVA and VMA.

Even though VMA and HVA are structurally related compounds with very similar characteristics, obtained *EF*s with the use of pentyl benzoate, hexyl benzoate, isoamyl benzoate and 1-octanol demonstrate differences in their HF-LPME behaviour (Fig. 11).

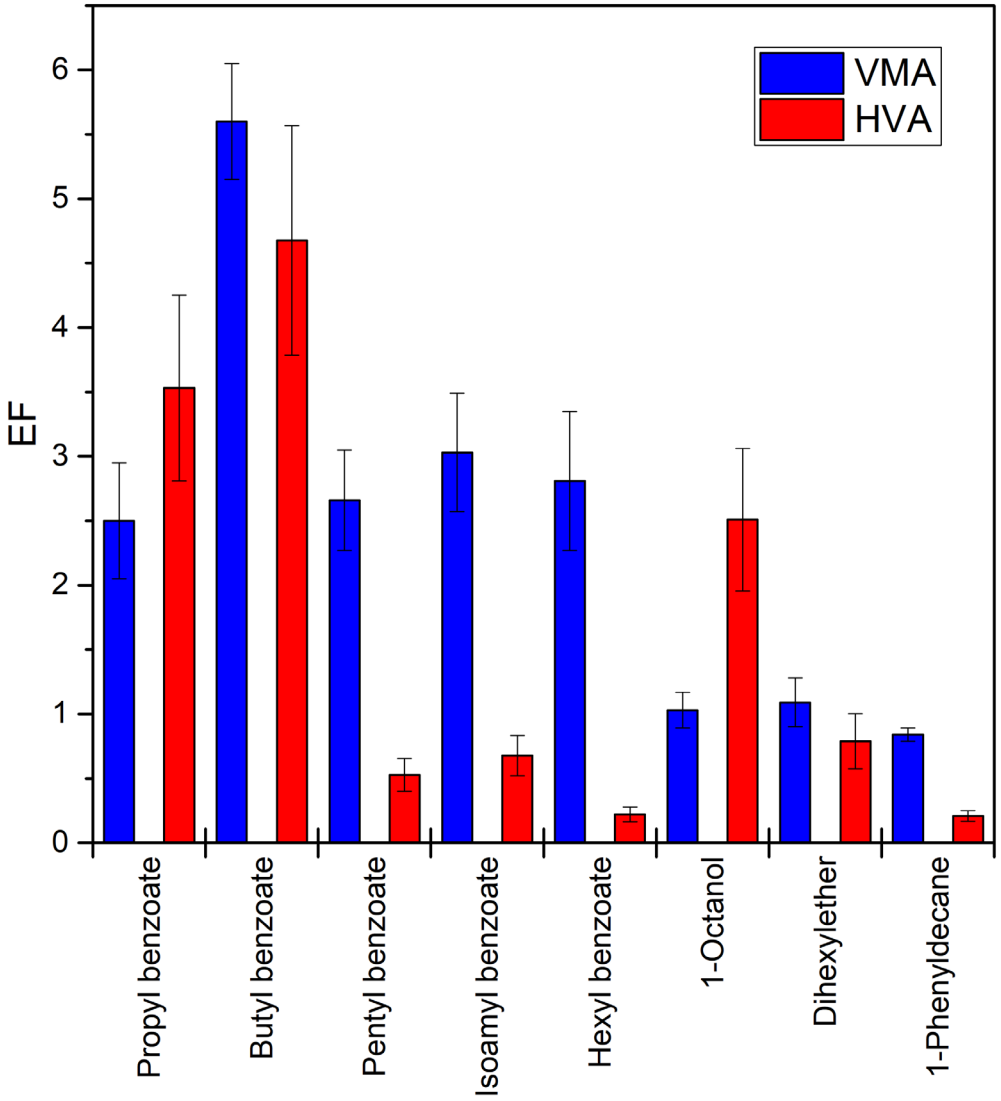

Fig. 11 *Enrichment factors (EF) of tested SLMs for HF-LPME-DPV of HVA and VMA. Acceptor solution: 0.1 mol* L^{-1} *NaOH,* V_{AS} *10 µL; Donor solution: 50 µmol* L^{-1} *of HVA or VMA in 0.1 mol* L^{-1} *HCl,* V_{DS} *10 mL; Extraction time 30 min; BDDE pre-treatment:* E_{act} *= −1.0 V,* t_{act} *= 15 s. The coverage intervals are on the significance level* $\alpha = 0.05$.

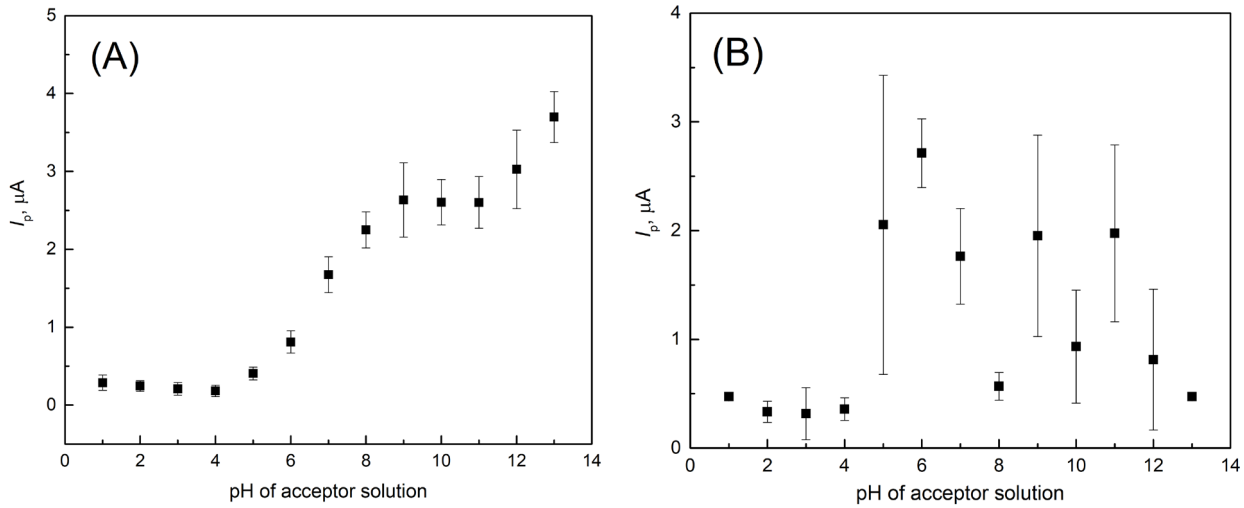

Fig. 12 *Dependence of DPV peak current of VMA (A) and HVA (B) after HF-LPME on pH of acceptor solution. Donor solution: 50 µmol L^{-1} VMA (A) or HVA (B) in 0.1 mol L^{-1} HCl, V_{DS} = 10 mL. Acceptor solutions: 0.1 mol L^{-1} PBS with constant ionic strength I = 0.55 mol L^{-1}, 1 mol L^{-1} HCl, 0.1 mol L^{-1} HCl, or 0.1 mol L^{-1} NaOH, V_{AS} = 10 µL. SLM: butyl benzoate, t_{ext} = 30 min; BDDE pre-treatment: E_{act} = −1.0 V, t_{act} = 15 s.*

Next, the effect of pH of the donor and acceptor solutions was investigated. The highest extraction efficiencies were presumed to be recorded under acidic conditions of donor solution and with basic acceptor solution, which allow most favourable partition coefficient of HVA and VMA between HF-LPME phases and maximum entrapment of analytes in the acceptor solution. The effect of recorded peak current on the pH of donor solution was, as expected, a decrease of peak currents with rising pH. The optimum donor solution pHs for both HVA and VMA were set to 1. Effect of acceptor solution was more intricate and showed interesting differences between HVA and VMA HF-LPME behaviour (Fig. 12). For VMA, biggest HF-LPME-DPV signals were recorded at pH 13 (Fig. 12A). In the pH range from 1 to 4, VMA was extracted even in its neutral form, although in a very limited way. This illustrates the influence of pH of donor and acceptor phase on the selectivity of extraction towards possible interferences. Two plateaus in the dependency correspond to the apparent dissociation constants of VMA, i.e., $pK_{a\,1} = 3.44$ and $pK_{a\,2} = 9.93$ [47]. In the case of HF-LPME-DPV of VMA, the efficiency of the extraction was more decisive factor than dependence of VMA peak current on pH.

The situation was different for HVA (Fig. 12B). The largest HF-LPME-DPV peak currents were recorded with acceptor solution pH 6. The dependence is more irregular due to

complicated relationship between HVA voltammetric and HF-LPME behaviour, which are affecting the HF-LPME-DPV in opposite ways. HVA DPV peak decreases with rising pH. At the same time, high pH of acceptor solution is most favourable from the perspective of HF-LPME.

Afterwards, the effect of extraction time (t_{ext}) was studied in the range from 5 to 90 min. For both HVA and VMA, the optimum t_{ext} was set to 30 min, as the best compromise between recorded HF-LPME-DPV peak currents and their repeatability. HF-LPME-DPV concentration dependencies were constructed under the optimum conditions (Table 2) in the ranges from 0.25-100 (HVA) and 0.5-100 (VMA), obtained *LOQ*s and *LOD*s were 1.2/0.4 μmol L^{-1} for HVA and 1.7/0.5 μmol L^{-1} for VMA, respectively.

Table 2 *Optimum conditions and figures of merit for HF-LPME-DPV determination of HVA and VMA. BDDE working electrode was cathodically pre-treated,* $E_{act} = -1.0$ *V,* $t_{acc} = 15$ *s.*

Analyte	SLM	Donor phase	Acceptor phase	Time of extraction	Linear range [μmol L^{-1}]	*LOQ* [μmol L^{-1}]	*LOD* [μmol L^{-1}]
HVA	Butyl benzoate	0.1M HCl	0.1M phosphate buffer pH 6	30 min	0.4-100	1.2	0.4
VMA	Butyl benzoate	0.1M HCl	0.1M NaOH	30 min	0.5-100	1.7	0.5

Finally, the developed methods were applied for the determination of VMA (Fig. 13A) or HVA (Fig. 13B) in human urine samples. In contrast to direct voltammetry, both analytes provided analytically usable peaks under their respective optimum experimental conditions (Table 2). A small number of studied analytes was found by method of standard addition. The found concentrations were 10.12 ± 0.82 (8.1 %) μmol for VMA and 13.5 ± 1.3 (9.3 %) for HVA, respectively. The number in brackets corresponds to RSD for 5 repeated HF-LPME-DPV experiments. Application of calibration curve for these analyses yielded comparable results with much lower repeatability due to complicated matrix of the urine sample; the RSDs were over 17 %. In retrospect, even the errors of ~ 9 % were reached only thanks to

careful optimization of multiple factors of both HF-LPME and single drop DPV. The overall sensitivity and repeatability of these methods are still adequate for their intended use as a screening tests for neuroblastoma, as the reported statistically significant increase of HVA and VMA in urine is over 20 µmol L^{-1} [37, 39].

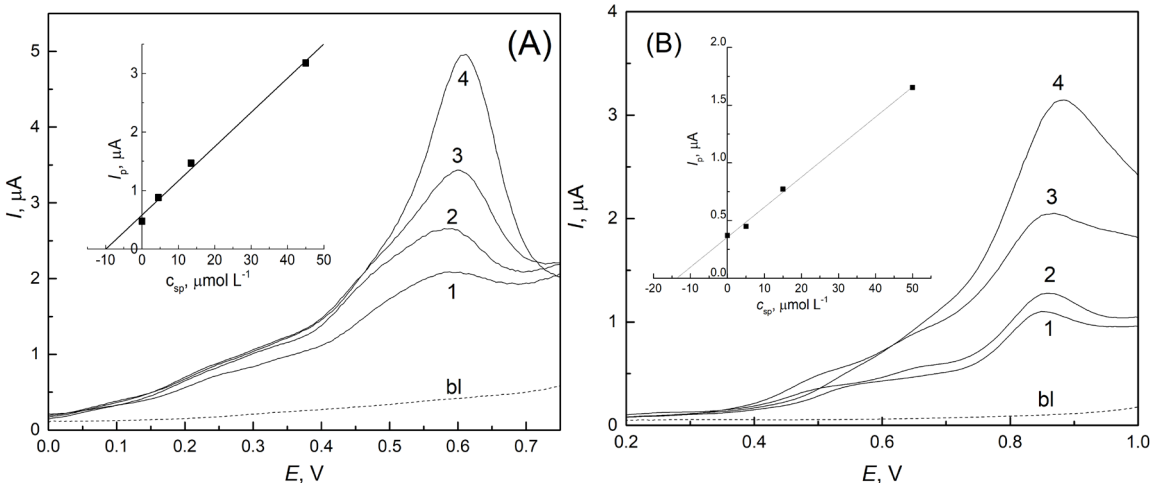

Fig. 13 *HF-LPME-DPV determination of VMA (A) HVA (B) in urine samples after HF-LPME. Curves: urine (1); urine with additions of HVA/VMA standards to concentration of 5 µmol L^{-1} (2), 15 µmol L^{-1} (3), and 50 (4) µmol L^{-1}. Dotted curve: extraction from donor solution in absence of urine and HVA/VMA addition. Donor solution: Centrifuged mixture of 1 mL of 1 mol L^{-1} HCl and 9 mL of urine, without or with addition of the standard. Other conditions are same as in Table 2. Insets: plot of standard additions, concentration values correspond to the real dilution.*

3.2 DMPS

This chapter serves mostly as a summary of publication **[2]** with expanded discussion of interesting or unexpected results.

3.2.1 Voltammetric determination of DMPS

The initial aim of the study was the development of a new voltammetric method for the determination of DMPS with the use of polished (p-AgSAE) and mercury meniscus modified solid silver amalgam electrode (m-AgSAE). The optimization was a simple one-factor-at-a-time method for both electrodes.

The effect of pH was investigated using cathodic stripping voltammetry (DCCSV) in the range from 1 to 13. Optimum pHs with the highest and the best developed peaks were set to 10 for p-AgSAE and 5 for m-AgSAE. Dependence of the peak potential on pH was Nernstian from pH 1 to 10 with slope −59 mV pH^{-1} at p-AgSAE and −51 mV pH^{-1} at m-AgSAE, respectively, in accordance with DMPS acid dissociation constants [109, 143]. This also confirmed proton coupled electron transfer corresponding to the reduction of the metal-thiolate.

Next, the cleaning procedure was optimized. Repeatability of no pre-treatment, mechanical polishing, application of regeneration potentials, and the use of CV were tested and compared. No pre-treatment/cleaning has led to pronounced passivation, as the peak currents gradually decreased by 50 % within only five repeated scans. The optimum cleaning procedure for p-AgSAE in BRB pH 10 was the application of 10 CV scans from −0.2 V to −1.8 V, ν = 0.5 V s^{-1} before each experiment. Analogously, the optimum cleaning procedure for m-AgSAE in BRB pH 5 was 5 CV scans in the range from 0 to −1.0 V. Regeneration potentials were tested in various ranges, but didn´t provide improved results over the cleaning procedures based on CV. Polishing has led to unacceptably high errors, expressed as relative standard deviation, RSD > 19 % (n = 10).

The optimization of potential (E_{acc}) and time (t_{acc}) of accumulation followed. The optimum E_{acc} and t_{acc} were −0.2 V/30 s for p-AgSAE and 0.0 V/15 s for m-AgSAE. These values were a compromise between the largest obtained peak currents and the repeatability.

The optimum conditions were used to obtain DPCSV concentration dependencies in a range of DMPS concentrations from 0.1 to 10 μmol L^{-1}. At p-AgSAE, the dependency was linear in the range from 0.26 to 2 μmol L^{-1} and logarithmical from 2 to 10 μmol L^{-1}. *LOQ* and *LOD* were 0.78 and 0.26 μmol L^{-1}, respectively. At m-AgSAE, the dependency was approximately linear in two distinctive regions, from 0.1 to 1 μmol L^{-1} and 1 to 10 μmol L^{-1}.

The developed method was applied for analysis of DMPS in human urine and in drug Dimaval (Table 3). Both p-AgSAE and m-AgSAE provided good recoveries and repeatability, except for analysis of urine at p-AgSAE with corresponding RSDs of 7.8 % and 12.6 % for added DMPS concentration of 10 μmol L^{-1} and 1.0 μmol L^{-1}. It´s notable, that the optimization and fine tuning of cleaning procedure, E_{acc} and t_{acc}, often at the expense of overall sensitivity is considerably less complicated with the use of a mercury working electrode that can create a completely new surface for each experiment.

Table 3 *Validation parameters for DPCSV determinations of DMPS at m-AgSAE and p-AgSAE in model samples of urine and in drug Dimaval. Conditions used for p-AgSAE were pH 10, E_{acc} = − 0.2 V and t_{acc} = 30 s. Conditions used for m-AgSAE: pH 5, E_{acc} = − 0.0 V and t_{acc} = 15 s* [2].

	DMPS added, μmol L^{-1}	DMPS found, μmol L^{-1}	Recovery, %	RSD, %
		Dimaval		
p-AgSAE	1.0	0.940 ± 0.021	94.0 ± 2.1	2.6
	10.0	9.77 ± 0.16	97.7 ± 1.5	1.8
m-AgSAE	1.0	1.020 ± 0.013	102.0 ± 1.3	1.5
	10.0	10.190 ± 0.071	101.90 ± 0.71	0.8
		Urine		
p-AgSAE	1.0	1.072 ± 0.073	107.2 ± 7.3	7.8
	10.0	9.2 ± 1.0	92 ± 10	12.6
m-AgSAE	1.0	0.980 ± 0.045	97.7 ± 4.5	5.2
	10.0	10.27 ± 0.27	102.7 ± 2.7	2.6

3.2.2 Electrochemical and complexing behaviour of DMPS

As a natural progression of the study, additional properties of DMPS were studied. EVLS was used for additional elucidation of the electrode process and voltammetric titrations of DMPS by Pb^{2+} provided additional insight to its complexing behaviour from the perspective of electrochemistry.

The I_p/ν dependence DMPS reduction peak was a direct proportion on both p-AgSAE and m-AgSAE, confirming electrode processes controlled by adsorption. EVLS provided additional insight into the electrode reactions. Used reference scan rates were 0.32 V s^{-1} for m-AgSAE and 0.64 s^{-1} for p-AgSAE, respectively.

At m-AgSAE EVLS split the DMPS cathodic peak into two, corresponding to the successive reduction of the two thiol groups of DMPS with an additional preceding kinetic process. The situation was similar on p-AgSAE. EVLS identified two consecutive reductions in adsorbed state. An anodic counter peak at m-AgSAE corresponds to the diffusion controlled process with preceding kinetic step. This is presumably the dissolution of the electrode with the limiting step of diffusional transport of DMPS towards the electrode surface.

Lastly, DPCSV and DPASV titrations were performed by gradual addition of 1.0 mmol L^{-1} $Pb(NO_3)_2$ into 100 μmol L^{-1} of DMPS in acetate buffer of pH 5. DPCSV uncovered several processes with regard to $[Pb^{2+}]$:[DMPS] ratio in the solution, including transmetalation of Hg(DMPS) to Pb(DMPS) reduction of Hg(DMPS) in adsorbed state, reduction of Pb(DMPS) in adsorbed state and reduction of free Pb^{2+}. The full scheme is available in publication **[2]**, pages 7-8. Obtained data suggest a presence of stable Pb:DMPS complex in 1:1 ratio, in contrast with another study no complex with 1:2 ratio was observed [120].

4. Conclusion

This work shows possibilities of HF-LPME-DPV for the determination of clinical biomarkers VMA and HVA in urine. The developed methods are simple, sensitive, cost-effective and environmentally friendly. The main purpose of HF-LPME was the suppression of interferences in urine in order to make subsequent DPV determination of VMA/HVA possible. The work also demonstrates different DPV and HF-LPME behaviour of structurally similar HVA and VMA, which can be used for their discrimination. Reached *LOQ*s and *LOD*s are more than sufficient for its intended purpose as a screening tool.

Additionally, it proves possibility of HF-LPME of highly polar compounds, although with lower extraction efficiencies.

In order to fully take advantage of HF-LPME with a voltammetric detection step, a more suitable analyte can be contemplated. HF-LPME-DPV of basic compounds from urine could lower the background of voltammograms [90]. Obtaining higher EFs might be easier with more hydrophilic compounds [91]. And finally, selecting an analyte with matching optimum conditions for HF-LPME as well as for the following voltammetric step can considerably improve the performance as well.

The second part presents newly developed methods for determination of heavy metal poisoning antidote DMPS at p-AgSAE and m-AgSAE; and verification of the methods on samples of human urine and drug Dimaval. Newly reached *LOQ*s of 0.8 μmol L^{-1} at p-AgSAE and 0.3 μmol L^{-1} at m-AgSAE are significantly lower than the *LOQ* in the only comparable study (41 μmol L^{-1})[124]. Moreover, additional information about the DMPS electrode mechanism was provided, the electrode reaction consisted of coupled proton/electron transfer with two consecutive reductions in adsorbed state, preceded by a kinetic process. Voltammetric titrations of DMPS by Pb^{2+} identified several processes including reductions in adsorbed state and transmetalation. This demonstrates the capabilities of amalgam electrodes for electroanalysis in complex biological matrices.

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6. Appendices

Publications

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Application of hollow fibre based microextraction for voltammetric determination of vanillylmandelic acid in human urine

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ABSTRACT

A new hollow-fibre microextraction method has been developed for the determination of a clinical biomarker vanillylmandelic acid (VMA) in human urine. The extraction was used in combination with differential pulse voltammetry (DPV) on cathodically pre-treated boron doped diamond electrode (BDDE). Butyl benzoate was found to be the optimum solvent for creation of supported liquid membrane. Optimum donor and acceptor phases were 0.1 mol L^{-1} HCl and 0.1 mol L^{-1} NaOH, respectively, optimum extraction time was 30 min. Linear range was from 0.5 to 100 μmol L^{-1} (r = 0.9989), with good repeatability (RSD 7.9% for 50 μmol L^{-1} VMA aqueous sample (n = 7)). The limit of detection (*LOD*) was 0.5 μmol L^{-1}. The method was successfully applied for the determination of VMA in real human urine sample using a standard addition method, obtained RSD being 8.1% (n = 5). The method is sufficiently selective and sensitive for its intended use as a screening tool for elevated concentration of VMA.

1. Introduction

Vanillylmandelic acid (VMA) is final metabolic product of catecholamine hormone epinephrine in human body [1] and a well-established biomarker of various diseases, which are linked to altered excretion of catecholamines [2]. Elevated levels of VMA in urine, blood, or cerebrospinal fluid can be found in patients with autism [3], post-traumatic stress disorder [4], or cancerous diseases of catecholamine producing glands, for instance tumors of adrenal medulla pheochromocytoma [5], tumors of neural crest [6], and neuroblastoma [7], while lowered levels are linked e.g. to depressions [8]. Determination of urinary VMA is used in screening [9], diagnosis [10], monitoring of disease progression [11], and drug dosage optimization [12]. In clinical practice, levels of VMA are often expressed as a ratio to creatinine concentration in urine, which is approximately constant for particular sex and age. Levels of urinary VMA in healthy adults and 1-year old infants expressed in molar concentration are comparable, being 20 ± 8.8 μmol L^{-1} and 17.1 ± 7.9 μmol L^{-1}, respectively, whilst the VMA to creatinine ratio is decreasing with age [13–15]. Reported urinary VMA cut-off level for positive diagnosis of neuroblastoma was set as over 2.5 standard deviations above mean concentration for particular age [16], i.e. to 34.3 μmol L^{-1} for 1-year old infants.

Various analytical methods for the determination of VMA based on liquid chromatography [5,17], gas chromatography [3], and capillary electrophoresis [18] have been reported. Determination of VMA in complex biological matrices often requires a sample preparation step, for instance solid phase extraction, which further increases already high operation costs of instrumental equipment. Electrochemical methods present a simple, fast, sensitive, and less expensive alternative for the determination of VMA [19–24]. Due to the presence of phenolic group, VMA is electrochemically oxidizable on various electrode materials, with reported wide linear ranges over two orders of magnitude and submicromolar limits of detection, well below normal urinary VMA levels [25]. However, direct voltammetric or amperometric determination of VMA in biological matrices is often impossible due to electrochemically active interferences, presence of high-molecular species, which can be adsorbed on the electrode surface, and other undesirable matrix effects. In order to fully exploit the advantages of electroanalytical methods, they can be coupled with a suitable sample clean-up (separation) procedure, such as hollow fibre based liquid/liquid/liquid phase microextraction (HF-LPME) [26].

HF-LPME is a relatively new method for biological sample preparation, combining preconcentration and sample clean-up in one step [27]. Three-phase HF-LPME is used for extraction of acidic [28,29] or basic [30] analytes which are extracted from a stirred aqueous donor solution through a supported liquid membrane (SLM) to the acceptor

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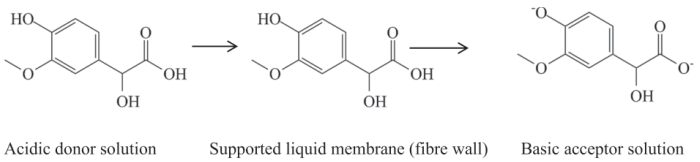

Scheme 1. Scheme of three-phase liquid/liquid/liquid hollow fibre microextraction of VMA.

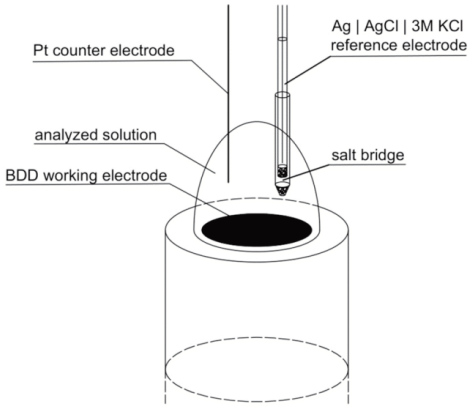

Fig. 1. Scheme of the used miniaturized 3-electrode system.

solution (Scheme 1). pH of used donor solution is adjusted so that the analytes of interest are present in their neutral form, which leads to higher solubility of the analytes in the SLM than in the donor solution. The SLM is typically used in the form of a water-immiscible organic solvent, which is immobilized in particular pores of polypropylene hollow fibre. For HF-LPME of polar analytes, octanol or aliphatic ethers are used as SLMs most frequently [31]. From such SLM, the analyte is transported to the aqueous acceptor solution inside the fibre lumen. The pH of the acceptor solution is adjusted to achieve maximum possible ionization and solubility of the analyte to suppress reverse transport. When buffered properly, donor solution can be analyzed by voltammetric techniques without any additional treatment [26]. Efficiency and selectivity of the process depends strongly on the partition coefficient of the analyte in the used SLM. Due to negligible solvent consumption and non-toxicity of fibre materials, HF-LPME is considered as more environmentally friendly ("green") than traditional extraction methods, such as solid phase or liquid/liquid extractions in larger volumes. HF-LPME and related methods have been thoroughly described in recent reviews [27,31,32].

The aim of this study is to develop a rapid, inexpensive and automatable method for the determination of VMA in human urine for preliminary screenings for neuroblastoma using hollow fibre based microextraction coupled with differential pulse voltammetry.

2. Experimental

2.1. Chemicals

Stock solution ($c = 1{\cdot}10^{-3}$ mol L^{-1}) of VMA (α,4-dihydroxy-3-methoxybenzeneacetic acid, CAS number: 3695-24-7, ≥98%, Sigma Aldrich, Czech Republic) was prepared by dissolving appropriate amount of the substance in deionized water (conductivity < 0.05 μS cm^{-1}, GORO system, Czech Republic). Stock solutions were kept in cold and dark. Methyl benzoate (99%, p.a.), ethyl benzoate (≥99%, p.a.), propyl benzoate (99%, p.a.), butyl benzoate (99%, p.a.), isoamyl benzoate (≥98%, food grade), pentyl benzoate (CPR grade), hexyl benzoate (food grade); 1-octanol (99%, p.a.), dihexyl ether (97%, p.a.),1-phenyldecane (98%, p.a.), L-ascorbic acid (99%), and uric acid (≥99%) were all purchased from Sigma Aldrich, Czech Republic. Acetone, hydrochloric acid (35%), phosphoric acid (85%), sodium hydroxide, and sodium chloride (all p.a.) were obtained from Ing. Petr Švec - PENTA, Czech Republic. Phosphate buffer system was prepared by mixing appropriate amounts of 0.2 mol L^{-1} NaOH with 0.1 mol L^{-1} phosphoric acid, ionic strength was set to 0.55 mol L^{-1} by addition of proper amount of NaCl. The pH of the buffer solution was measured with a pH meter Jenway 3505 (Jenway, United Kingdom) with a combined glass electrode. Q3/2 Accurel polypropylene hollow fibre (600 μm i.d., 200 μm wall thickness, and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany).

2.2. Apparatus

Voltammetric measurements were realized by Eco-Tribo-Polarograph (ETP) (Polaro-Sensors, Czech Republic). This device was controlled by a desktop computer with operating system Microsoft Windows 7.1 (64 bits) (Microsoft, USA) and electrochemical software MultiElChem (version 3.1) (J. Heyrovský Institute of Physical Chemistry of the Czech Academy of Sciences, Czech Republic). Measurements were carried out in a miniaturized three-electrode system with upside-down arranged [33] boron doped diamond working electrode (BDDE, 3 mm diameter, Windsor Scientific, UK), platinum wire counter electrode (diameter 0.28 mm, Monokrystaly, Czech Republic), and Ag|AgCl| (3 mol L^{-1} KCl) reference electrode (type 10–20+, Elektrochemické detektory, Czech Republic) connected with the measured solution by a salt bridge (filled with 0.1 mol L^{-1} NaOH) with small frit ending (S2 frit, diameter 1.0 mm) (Fig. 1). All below given potential values are referred to the used reference electrode.

2.3. Procedures

Q3/2 Accurel polypropylene hollow fibre (600 μm i.d., 200 μm wall thickness, and 0.2 μm pore size) was cut into 20 mm long pieces, rinsed with acetone in an ultrasonic bath for 5 min, and dried. Prior to an experiment, the hollow fibre was soaked in an organic solvent for 5 s to impregnate the pores. Excess of the organic solvent was pushed out from the fibre by air from a syringe. The prepared fibre was consequently filed with 10 μL of acceptor phase, and both ends of the fibre were sealed with custom-made glass plugs. The extraction (Fig. 2) was performed by immersing the sealed fibre fully into the donor solution, which was stirred by magnetic stirrer (AREC.X, P-LAB, Czech Republic) with Teflon stir bar (2 cm, P-LAB, Czech Republic). Stirring rate was set to 1500 rpm. After the extraction, the fibre was immediately taken out from the donor solution, unplugged, and the acceptor phase was withdrawn from the fibre and transferred by clean syringe to the working electrode surface for voltammetric analysis. DPV was carried out at the scan rate $\nu = 20$ mV s^{-1}, pulse height 50 mV, pulse width 80 ms followed by sampling time 20 ms. Samples of urine were centrifuged at Eppendorf MiniSpin (Eppendorf, Czech Republic) for 5 min and stored in cold and dark. Each experiment was repeated five times and statistically evaluated, confidence intervals were calculated on the

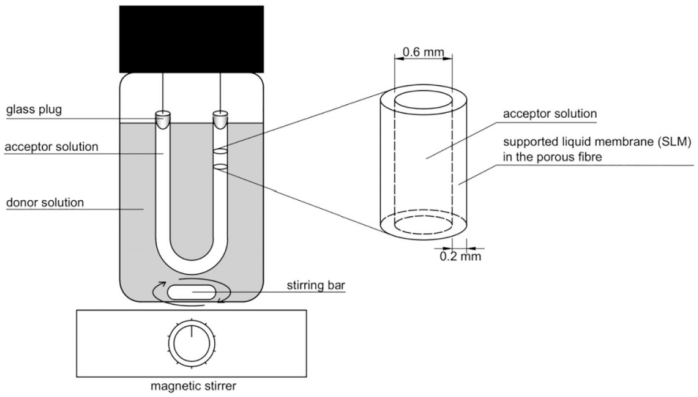

Fig. 2. Scheme of experimental setup used for hollow fibre based liquid phase microextraction.

level of significance $\alpha = 0.05$, including error bars and confidence bands in graphs. Limits of detection and quantification were calculated using direct signal method according to IUPAC [34].

3. Results and discussion

3.1. Voltammetric determination of VMA at the BDDE

A method for VMA determination at anodically pre-treated BDDE was already published by our group [20], with reported optimum pH 3. However, under the basic conditions of hollow fibre acceptor solution, the repulsion between negatively charged VMA molecule and the $-O^-$ and $-COO^-$ groups on the O-terminated BDDE surface leads to pronounced shift of VMA oxidation DPV peak towards more positive potentials $E_p \approx +1200$ mV with poor repeatability of peak potentials and currents. Therefore, it was necessary to modify this method for alkaline conditions used by hollow fibre microextraction described in this manuscript. In order to obtain stable and sufficiently high peak currents, effect of BDDE pre-treatment procedure was investigated. Optimum potential of electrode activation under basic conditions of 0.1 mol·L^{-1} NaOH was found to be $E_{act} = -1000$ mV vs. Ag|AgCl|3 M KCl, more negative activation potentials have led to gradually rising background current and lower repeatability. Activation time was set to $t = 15$ s, since longer activation times provided no additional improvement. End potential of the voltammetric scan was set to $E_{fin} = +750$ mV to avoid undesirable oxidation and change of termination of the BDDE surface. Two well-developed VMA peaks were recorded at +260 and + 580 mV vs. Ag|AgCl|3 M KCl. Presumably, they correspond to the mechanism of electrochemical oxidation of VMA on carbon electrodes proposed by Li et al. [35].

For all further experiments, including concentration dependencies, the second, larger peak was used. Under the optimized conditions using the miniaturized three electrode system with sample volume 10 µL, ten repeated measurements of 50 µ mol L^{-1} VMA in 0.1 mol L^{-1} NaOH provided stable current responses with RSD = 5.9%. The use of a conventional electrode system with 10 mL sample volume provided RSD = 1.9% ($n = 10$), proving that the increasing RSD is caused mainly by the miniaturization rather than by inadequate experimental conditions. Linear dependencies of the DPV peak current on the VMA concentration were obtained in the concentration range from 1 to 100 µmol L^{-1} (Table 1), corresponding voltammograms are shown in Fig. 3. *LOQ* and *LOD* were 3.3 µmol L^{-1} and 1.0 µmol L^{-1}, respectively, i.e. sufficient for the intended purpose as a preliminary screening tool for elevated levels of VMA.

Moreover, the effect of two major urinary interferences, ascorbic acid (AA) and uric acid (UA), was investigated. Due to large potential difference between second VMA peak and AA and UA peaks, influence of AA and UA on the VMA peak current proved to be negligible, as shown in Fig. 4. However, in the presence of real urine in the analyzed sample, VMA signal is completely overlapped by the background, even with additions of VMA up to overall concentration 1000 µmol L^{-1}. Therefore, insertion of a suitable sample preparation step is essential.

3.2. Optimization of the HF-LPME procedure

Hollow fibre length was set to 20 mm long segments to provide sufficient acceptor solution volume of 10 µL for the subsequent voltammetric detection. Used donor solution volume was 10 mL to ensure complete immersion of the hollow fibre, easy manipulation, and adequate stability of the system. Contrary to solid-phase extraction, volume of HF-LPME donor phase does not affect the extraction efficiency, as long as the donor solution volume is kept significantly higher than acceptor solution volume. Stirring of the donor solution is essential for reducing thickness of boundary layer at the outer membrane surface and faster transport of the analyte. Majority of our experimental work was aimed at optimization of the most important HF-LPME parameters: composition of the supported liquid membrane, pH of both donor and acceptor phase and extraction time.

3.2.1. Supported liquid membrane composition

For HF-LPME of VMA, various organic solvents have been tested as supported liquid membranes. Selection was limited to solvents with low viscosity, negligible miscibility with aqueous solutions, low volatility, no electrochemical interference in the selected potential range, and relatively high polarity in order to get the largest possible partition coefficient of VMA between SLM and donor phase. Some of these requirements, such as polarity and water immiscibility are contradictory to each other; experimental optimization is therefore necessary. Tested solvents included three most commonly used ones for LMPE of polar acidic analytes [31]: 1-octanol, dihexyl ether, and 1-phenyldecane. Moreover, according to the *"similia similibus solvuntur"* rule, a whole sequence of benzoic acid esters from methyl benzoate to hexyl benzoate was also used. Concentration of VMA in the donor solution was initially set to 50 µmol L^{-1} as an approximate value of elevated VMA levels in urine.

Benzoates proved to be well suitable as SLMs for HF-LPME of VMA. We have found two exceptions among them only: methyl benzoate and ethyl benzoate, which provided very unstable VMA peak potentials and

Table 1
Parameters of concentration dependencies for DPV determination of VMA at cathodically pre-treated BDDE in 0.1 mol L^{-1} NaOH with sample volume 10 µL.

Concentration range (µmol L^{-1})	Slope (mA·mol^{-1})	Intercept (nA)	Correlation coefficient	*LOQ* (µmol L^{-1})	*LOD* (µmol L^{-1})
10–100	19.93 ± 0.16	24 ± 10	0.9996	–	–
1–10	24.27 ± 0.64	−28.6 ± 4.1	0.9971	3.3	1.0

peak currents with RDS over 100%. It was caused probably by their higher solubility in water resulting in lower stability in the fibre.

Enrichment factor was calculated as a ratio of final VMA concentration in acceptor phase to initial VMA concentration in the donor solution. The highest enrichment factor (5.60 ± 0.45) was obtained using butyl benzoate (Table 2) (Fig. 5). Hence, it was selected as the optimum SLM. Butyl benzoate SLM also provided very good peak current repeatability with RSD = 7.9%. Contrary to the DP voltammograms obtained without extraction (Fig. 3), first VMA peak at +280 mV is practically non-apparent after extraction using butyl benzoate as SLM (Fig. 5). Although 1-phenyldecane SLM provided lower enrichment factor for VMA extraction, high repeatability of the peak current could be potentially exploited for its use as a SLM for extraction of less polar analytes.

3.2.2. Effect of pH of donor and acceptor solutions

Composition of donor and acceptor solution has significant effect on the extraction efficiency. Therefore, it had to be carefully optimized and controlled. Initially, pH of donor and acceptor solutions was set to 1 and 13, respectively (using 0.1 mol L^{-1} HCl and 0.1 mol L^{-1} NaOH), with respect to VMA acidic dissociation constants, i.e., $pK_{a\,1}$ = 3.44 and $pK_{a\,2}$ = 9.93 [36]. VMA was expected to be transported in its neutral form from the donor solution to the SLM and entrapped in the acceptor solution in its dissociated form. This presumption was confirmed (see Fig. 6). It corresponds to the effects of pH of acceptor and donor solutions on VMA peak current. In order to minimize the effect of ionic strength of buffer solutions used for different pH values, phosphate buffer with constant ionic strength set to I = 0.55 mol L^{-1} was used for pH 2–12. Concerning pH of acceptor solution, the highest VMA DPV peak currents were obtained in the samples with higher pH of the acceptor phase. Finally, 0.1 mol L^{-1} NaOH was therefore selected as optimum. Two plateaus in Fig. 6A reflect presence of VMA in different forms with respect to its apparent dissociation constants. Donor solution pH also affected the VMA peak current as previously expected (Fig. 6B), as the peak current was increasing with decreasing pH of the donor solution. However, the use of HCl of concentration 1 mol L^{-1} and higher has led to poorer repeatability and surprisingly slightly lower peak currents in comparison with 0.1 mol L^{-1} HCl. Thus, 0.1 mol L^{-1} HCl was selected as the optimum donor phase.

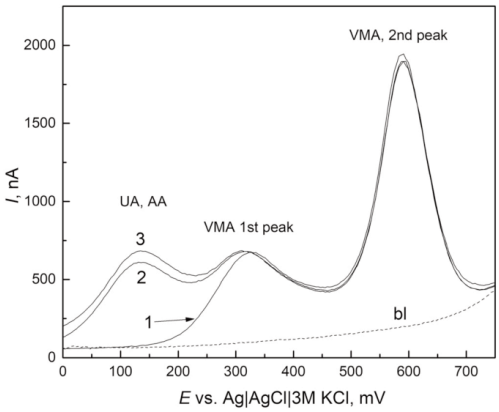

Fig. 4. DP voltammograms of 100 µmol L^{-1} VMA (1), 100 µmol L^{-1} VMA + 100 µmol L^{-1} AA (2), and 100 µmol L^{-1} VMA + 100 µmol L^{-1} AA +100 µmol L^{-1} UA (3) at BDDE in 0.1 mol L^{-1} NaOH, sample volume 10 µL (without any prior extraction). Dotted curve corresponds to the blank sample (bl). BDDE was pre-treated at E_{act} = −1000 mV for t = 15 s.

3.2.3. Effect of extraction time

The dependency of peak current on the extraction time was investigated in the range from 5 to 60 min (Fig. 7). VMA DPV peak currents increased with increasing extraction times. However, RSD of five repeated experiments was higher than 20% for extraction times exceeding 30 min. Possible explanation is a loss of organic solvent from SLM due to its gradual dissolution in the donor phase or even its partial evaporation. Thus, 30 min were set as optimum extraction time, providing high extraction efficiency with satisfactory repeatability.

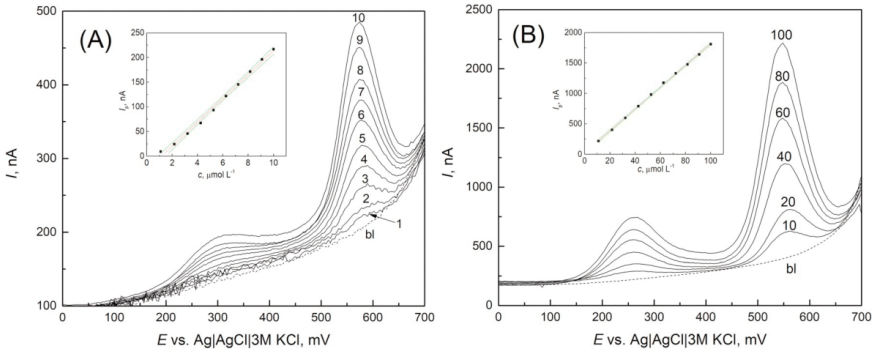

Fig. 3. DP voltammograms of VMA at BDDE in 0.1 mol L^{-1} NaOH, sample volume 10 µL, VMA concentration range from 1 to 10 µmol L^{-1}(A) and 10 to 100 µmol L^{-1}(B) (without any prior extraction). The numbers next to the curves correspond to the analyte concentrations in µmol L^{-1}. The dotted curve corresponds to the blank sample (bl). BDDE was pre-treated at E_{act} = −1000 mV for t = 15 s. Inset: Dependence of VMA peak current (at 580 mV) on the VMA concentration.

Table 2
Comparison of SLMs for HF-LPME of VMA. Donor solution 10 mL of 50 μmol L^{-1} VMA in 0.1 mol L^{-1} HCl, acceptor solution 10 μL of 0.1 mol L^{-1} NaOH. Time of extraction 30 min. DPV measured at cathodically pre-treated BDDE, $E_{act} = -1000$ mV, $t_{act} = 15$ s.

SLM	Enrichment factor	RSD (%)
Methyl benzoate	–[a]	
Ethyl benzoate	–[a]	
Propyl benzoate	2.50 ± 0.45	18.2
Butyl benzoate	5.60 ± 0.45	7.9
Pentyl benzoate	2.66 ± 0.39	14.7
Isoamyl benzoate	3.03 ± 0.46	15.3
Hexyl benzoate	2.81 ± 0.54	19.4
1-Octanol	1.03 ± 0.14	13.9
Dihexyl ether	1.09 ± 0.19	17.5
1-Phenyldecane	0.84 ± 0.05	5.9

[a] Poor repeatability of VMA peak potentials and currents, RSD > 100%.

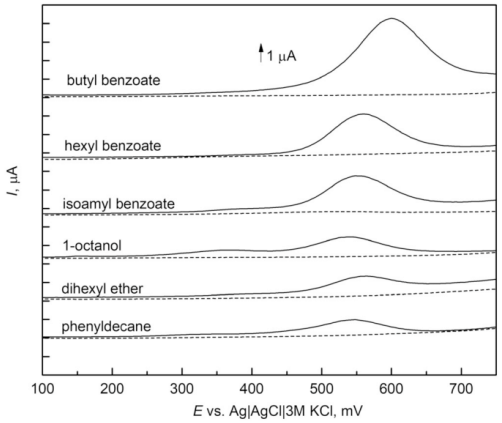

Fig. 5. DP voltammograms of VMA after HF-LPME using various supported liquid membranes. Donor solution 10 mL of 50 μmol L^{-1} VMA in 0.1 mol L^{-1} HCl, acceptor solution 10 μL of 0.1 mol L^{-1} NaOH, extraction time 30 min, hollow fibre length 20 mm. BDDE was pre-treated at $E_{act} = -1000$ mV for $t = 15$ s.

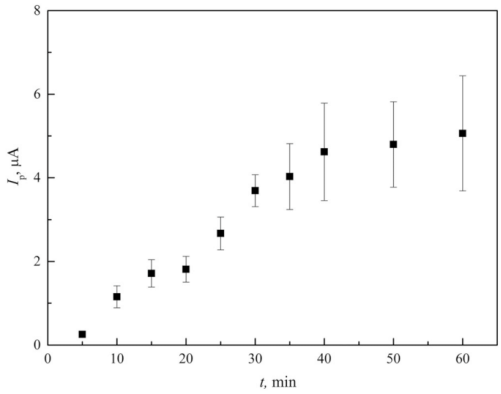

Fig. 7. Effect of HF-LPME time on VMA DPV peak current. Extraction times 5–60 min. Donor solution: 10 mL 50 μmol L^{-1} VMA in 0.1 mol L^{-1} HCl, acceptor solution: 10 μL 0.1 mol L^{-1} NaOH, SLM butyl benzoate, hollow fibre length 20 mm. BDDE was pre-treated at $E_{act} = -1000$ mV for $t = 15$ s.

3.3. Concentration dependency of DPV of VMA after HF-LPME

Dependency of the peak current on VMA concentration in donor solution was investigated under established optimum extraction conditions in the range from 0.5 to 100 μmol L^{-1}. The dependency was linear in the whole investigated range. Corresponding DP voltammograms in the concentration range 10–100 μmol L^{-1} and 0.5–10 μmol L^{-1} are shown in Fig. 8. For the sake of optimum accuracy, peak currents were evaluated from the line connecting linear parts of DP voltammograms before onset and after the end of the peak, rather than from the blank curve (due to less stable background of the DP voltammograms). Parameters of recorded calibration straight line are summarized in Table 3 with $LOD = 0.5$ μmol L^{-1} and $LOQ = 1.7$ μmol L^{-1}.

3.4. Analysis of VMA in human urine

The newly developed method was applied to human urine samples, provided by healthy volunteer (28 years old male). Donor solution was

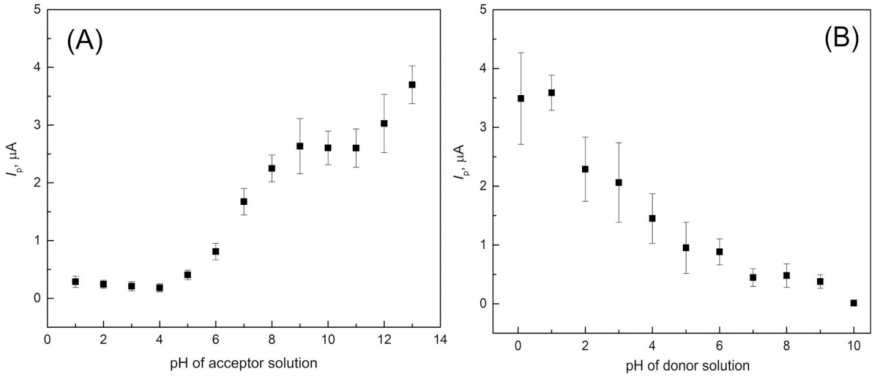

Fig. 6. Effect of pH of HF-LPME acceptor (A) and donor (B) solution pH on VMA DPV peak current. (A) Donor solution: 10 mL of 50 μmol L^{-1} VMA in 0.1 mol L^{-1} HCl, acceptor solution pH: 1–13. (B) Acceptor solution: 10 μL 0.1 mol L^{-1} NaOH, donor solution 50 μmol L^{-1} VMA, pH: 0–10. Donor and acceptor solutions of various pH were phosphate buffers with constant ionic strength $I = 0.55$ mol L^{-1}, 1 mol L^{-1} HCl, 0.1 mol L^{-1} HCl, or 0.1 mol L^{-1} NaOH; SLM butyl benzoate, extraction time 30 min. Hollow fibre length 20 mm. BDDE was pre-treated at $E_{act} = -1000$ mV for $t = 15$ s.

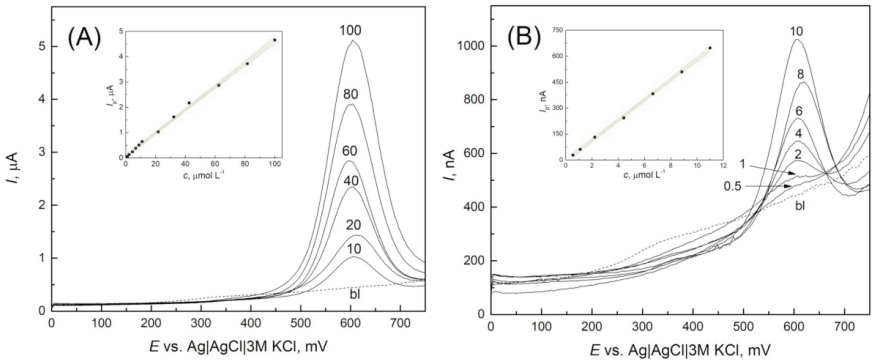

Fig. 8. DP voltammograms of VMA after HF-LPME. VMA concentrations from 10 to 100 µmol L^{-1} (A) and 0.5 to 10 µmol L^{-1} (B) in 0.1 mol L^{-1} HCl donor solution (concentration values are presented next to the corresponding curves). Dotted curve (bl) corresponds to the blank extraction from 0.1 mol L^{-1} HCl. Acceptor solution 10 µL of 0.1 mol L^{-1} NaOH, SLM butyl benzoate, extraction time 30 min. Hollow fibre length 20 mm. BDDE was pre-treated at $E_{act} = -1000$ mV for $t = 15$ s. Inset: dependence of peak current on the VMA concentration in donor solution.

prepared by adding 1 mL of 1 mol L^{-1} HCl to 9 mL of centrifuged non-diluted urine. HF-LPME-DP voltammograms of urine sample and urine samples with standard addition of VMA resulting in its concentration of 5, 15, and 50 µmol L^{-1} in particular urine sample before addition of HCl are shown in Fig. 9.

The VMA peak is sufficiently well developed and analytically usable in both spiked and non-spiked urine samples. Contrary to deionized water samples (Fig. 8), voltammetric background of urine samples is greatly increased by the presence of large amount of various electrochemically oxidizable interferences of acidic character, which are extracted simultaneously with VMA. However, the background is sufficiently flat and uniform for VMA peak evaluation. It was found that urine sample of the healthy volunteer contained small amount of VMA. Its concentration was evaluated using standard addition method and calibration curve method and it was found to be 10.12 ± 0.82 µmol L^{-1} and 10.2 ± 1.8 µmol L^{-1}, respectively (Table 4).

The standard additions provided more repeatable results than calibration curve method only. Higher error of the calibration curve is apparently caused by unstable background and subsequent less reliable peak evaluation, especially for lower concentrations. Nevertheless, overall sensitivity and selectivity of the method is more than adequate for the intended purpose of preliminary screenings for highly elevated urinary VMA in infants, since VMA levels in such samples are increased at least five-fold compared to healthy adults.

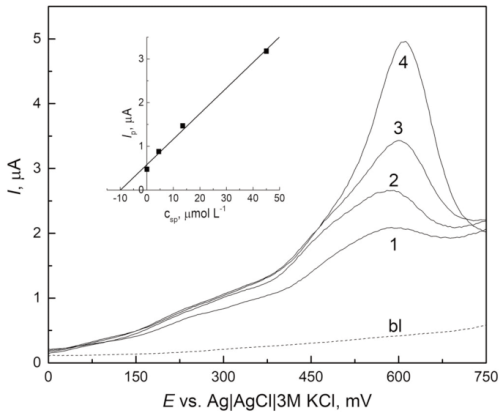

Fig. 9. HF-LPME-DP voltammograms of urine (1) and urine with standard additions of VMA to the concentration of 5, 15 and 50 µmol L^{-1}. Donor solution: 9 mL of centrifuged urine (with and without standard addition, respectively) + 1 mL 0.1 mol L^{-1} HCl. Dotted curve (bl) corresponds to blank extraction from 0.1 mol L^{-1} HCl donor solution. Acceptor solution 10 µL 0.1 mol L^{-1} NaOH, SLM butyl benzoate, extraction time 30 min. Hollow fibre length 20 mm. BDDE was pre-treated at $E_{act} = -1000$ mV for $t = 15$ s. Inset: standard addition plot with concentration values considering the real dilution.

4. Conclusions

A novel method of hollow fibre based liquid/liquid/liquid microextraction coupled with differential pulse voltammetry at boron doped diamond electrode for the determination of VMA was successfully applied to analysis of real urinary samples with sufficient selectivity and sensitivity. Repeatability can be possibly improved by automatic, rather than manual handling of very small volumes and hollow fibre preparation, as is the case for similar liquid microextraction methods [32]. Overall, the method is feasible as a large-scale high-throughput screening tool for elevated VMA levels in urine of the patients with

Table 4
Comparison of calibration curve and standard addition method for HF-LPME-DPV determination of VMA in urine sample of the healthy volunteer.

Method of evaluation	VMA concentration found (µmol L^{-1})	RSD (%)
Calibration curve	10.2 ± 1.8	17.7
Standard addition	10.12 ± 0.82	8.1

Table 3
Parameters of concentration dependency of HF-LPME-DPV determination of VMA.

Concentration range (µmol L^{-1})	Slope (mA·mol^{-1})	Intercept (µA)	Correlation coefficient	*LOQ* (µmol L^{-1})	*LOD* (µmol L^{-1})
0.5–100.0	4.57 ± 0.63	0.068 ± 0.027	0.9989	1.7	0.5

neuroblastoma and other relevant diseases. Other beneficial features of the method are simplicity, negligible solvent consumption and overall cost effectivity.

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Determination of heavy metal poisoning antidote 2,3-dimercapto-1-propanesulfonic acid using silver solid amalgam electrode

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ABSTRACT

Voltammetric behavior of heavy metal poisoning antidote 2,3-dimercapto-1-propane-sulfonic acid (DMPS) was investigated using linear scan voltammetry (LSV), differential pulse cathodic stripping voltammetry (DPCSV), differential pulse anodic stripping voltammetry (DPASV), and elimination voltammetry with linear scan (EVLS) at a polished (p-AgSAE) and at a meniscus modified (m-AgSAE) silver solid amalgam electrode. It was confirmed that the mechanism of the electrode process involves two consecutive reductive desorptions with coupled proton/electron transfer preceded by a kinetic process prominent at higher scan rates as revealed by EVLS. Voltammetric and complexation behavior of DMPS in the presence of Pb^{2+} was further investigated by DPASV and DPCSV titrations confirming reductive desorption, complex formation, and transmetalation. Optimum conditions for DPCSV were as follows: Britton-Robinson buffer (BRB) of pH 10, $E_{acc} = -200$ mV and $t_{acc} = 30$ s for p-AgSAE and BRB of pH 5, $E_{acc} = 0$ mV and $t_{acc} = 15$ s for m-AgSAE. Limits of quantification (*LOQs*) and detection (*LODs*) were 0.3 and 0.1 μmol L^{-1} at m-AgSAE and 0.8 and 0.3 μmol L^{-1} at p-AgSAE, respectively. The practical applicability of the newly developed method was verified by determination of DMPS in commercial drug Dimaval and in human urine samples.

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1. Introduction

DMPS (2,3-dimercapto-1-propanesulfonic acid, Fig. 1) is a synthetic dithiol antidote for intoxications by various heavy metals including lead, mercury, bismuth and arsenic [1,2]. The environmental and occupational exposure to lead continues to be a serious problem in developing countries, as 90% of lead can be stored in teeth and bones and later distributed to other organs including liver, kidney, or brain [3,4]. This can cause irreversible neurological problems, especially in infants [5]. DMPS is widely used for treatment of both acute and chronic lead exposure [6,7]. It has been applied as an antidote due to its strong complexing properties towards lead ions, low toxicity, negligible side effects, high water solubility, and ability to be administered orally, parenterally or intramuscularly [2,8]. DMPS-lead complex is excreted from human body through renal pathway [9]. Other notable applications of DMPS are in the treatment of Wilson's disease [10,11], amyloidosis [12], and diabetic polyneuropathy [13]. Sodium salt of DMPS is a drug registered as Dimaval, Unithiol, or Mercuval. For the sake of simplicity, the abbreviation DMPS will be used, when appropriate, for both sulfonic acid and its sodium salt.

Determination of urinary DMPS is relevant from the clinical point of view, since it can be utilized for monitoring of treatment and dose optimization to minimize excess heavy metal deposition in body tissue [14,15].

Voltammetric techniques are suitable for these purposes due to their high sensitivity, sufficient selectivity, speed, and low investment and operational costs [16]. Moreover, voltammetry can provide important information regarding electrode mechanism of DMPS and its complexation behavior. Several voltammetric studies of complexation properties of DMPS, dimercaptosuccinic acid, and other structurally analogous compounds towards heavy metal ions have been published, confirming the feasibility of DMPS

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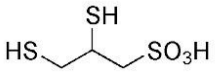

Fig. 1. Chemical structure of DMPS.

voltammetric determination, preferably alongside a heavy metal ion of interest [17–20]. DMPS voltammetric determination at multiwall carbon nanotube modified glassy carbon electrode [21] reported limit of quantification (*LOQ*) of 41 μmol L^{-1}. Silver solid amalgam electrode (AgSAE) was chosen in this work due to its ability to form complexes with thiols enabling the use of stripping voltammetry [22]. Thiol groups are oxidatively chemisorbed on AgSAE forming semi-covalent bonds with mercury or silver atoms even under open circuit conditions (Eq. (1)) [23]. Thiol deposition can be controlled by the application of external potential. This process can serve as an accumulation step for cathodic stripping voltammetry (CSV) [24]. In the stripping step, a reverse reaction described by (Eq. (1)) takes place.

$$Hg + R{-}SH \text{ - > } Hg{-}S{-}R + e^{-} + H^{+} \quad (1)$$

AgSAEs retain most of desirable properties of mercury electrodes such as high signal to noise ratio, wide potential window, and often submicromolar limits of detection (*LODs*) [25–27]. Silver solid amalgam (AgSA) is practically non-toxic, mechanically stable material containing no liquid mercury [28–31]. Furthermore, its surface can be easily and quickly electrochemically regenerated even in the presence of chemisorbed thiols, provided they are sufficiently soluble in water [22,32].

The aim of this work was to develop a new voltammetric method for the determination of DMPS at two different solid amalgam electrodes: polished (p-AgSAE) and mercury meniscus modified (m-AgSAE), including clarification of electrode mechanism using elimination voltammetry with linear scan [33] (EVLS). Furthermore, voltammetric titrations of DMPS by Pb^{2+} were performed to elucidate electrochemical aspects of complexation behavior of DMPS towards Pb^{2+}.

2. Experimental

2.1. Chemicals and instrumentation

The stock solution of 2.124 M DMPS was prepared by dissolving 10 mg of solid 2,3-dimercapto-1-propanesulfonic acid monohydrate, p.a. (Merck, Czech Republic) in 100 mL of deionized water using ultrasonic bath (P-LAB, Czech Republic). It was stored in dark at 5 ^{O}C. Britton-Robinson buffer (BRB) solutions of pH values from 2 to 12 were prepared by mixing the proper amounts of alkaline component of 0.2 M NaOH (Lachema, Czech Republic) and of acidic components consisting of 0.04 M H_3BO_3, 0.04 M H_3PO_4 and 0.04 M CH_3COOH (all Lachema, Czech Republic). The alkaline solution was prepared by dissolution of 3.995 g of NaOH, p.a., in 500 mL of deionized water and the acid solution was prepared by dissolution of 1.235 g of H_3BO_3, p.a., 0.88 mL of H_3PO_4 (85%), p.a., and 1.435 mL of CH_3COOH (99%), p.a., in 500 mL of deionized water. Other used chemicals were $Pb(NO_3)_2$, p.a., and HCl, p.a., (both Lachema, Czech Republic). For all the measurements, deionized water from Milli-Q-Gradient, Millipore, Prague, Czech Republic (conductivity <0.05 μS/cm) was used.

Voltammetric measurements were executed with the computer-controlled Eco-Tribo Polarograph (Polaro-Sensors, Prague, Czech Republic), equipped with MultiElChem 3.1 software for Windows XP/7/8/10 (J. Heyrovský Institute of Physical Chemistry of the Czech Academy of Sciences, Czech Republic). Two types of working electrode were used: m-AgSAE with the working surface of 0.382 ± 0.025 mm^2 (level of significance, $\alpha < 0.05$), and p-AgSAE with the working surface of 0.196 ± 0.015 mm^2 ($\alpha < 0.05$).

Ag|AgCl|3 M KCl was used as the reference and platinum wire (⌀ 1 mm) as the auxiliary electrode (both from Elektrochemicke detektory, Czech Republic). The measurements were performed at laboratory temperature (25 ± 2C). Oxygen was removed from the measured solutions by bubbling nitrogen (purity class 4.6; Messer Technogas, Prague, Czech Republic) for 5 min. The pH was measured using pH-meter Jenway 3505 using combined glass electrode, type 924 001 (Bibby Scientific Limited, UK).

Drug Dimaval (Heyl, Germany) was used for the preparation of model sample solution. One shelled hard capsule contains (according to the manufacturer's declaration) 121 mg of solid sodium monohydrate salt of DMPS, corresponding to 100 mg of DMPS. Stock solution with DMPS concentration of 0.531 mmol L^{-1} was prepared by dissolution of the entire content of one shell capsule in 1.0 L of deionized water. Two model samples of Dimaval solution (1.0 μmol L^{-1} and of 10 μmol L^{-1}) were prepared by addition of appropriate volume of ten-times diluted Dimaval stock solution into 10 mL of BRB.

Urine model sample was prepared by mixing a sample obtained from healthy 30 years old male volunteer with BRB in 1:1 ratio. Its pH was adjusted to proper value by addition of 0.2 mol L^{-1} NaOH. Afterwards, it was spiked with Dimaval stock solution to total concentrations of 1.0 μmol L^{-1} and 10 μmol L^{-1}.

2.2. Procedures

Purified nitrogen was passed through the analyzed solution for 5 min before each measurement, and then nitrogen atmosphere was maintained above the solution in the cell during the measurement. Before each set of experiments, p-AgSAE was renewed by polishing on polyurethane pad impregnated with Al_2O_3, particles size 0.5 μm. The electrode surface was activated by insertion of the cleaning potential E_{cl} of −2200 mV for 300 s in 0.2 mol L^{-1} KCl. Prior to each individual measurement, both p-AgSAE and m-AgSAE were activated by application of five cyclic voltammetric (CV) cycles from the initial potential E_{in} −200 mV to the final potential E_{fin} −1800 mV with a scan rate (υ) of 500 mV s^{-1} in the sample solution.

The parameters of the direct current voltammetry (DCV) and CV were as follows: $E_{in} = 0$ mV, $E_{fin} = -1800$ mV, and $\upsilon = 100$ mV s^{-1}. Differential pulse voltammetry (DPV) was carried out with the pulse width of 100 ms, sampling time of 20 ms at the end of the pulse, pulse height of −50 mV for cathodic scans and +50 mV for anodic scans, respectively, and υ of 20 mV s^{-1}. Unless stated otherwise, the accumulation step of direct current cathodic stripping voltammetry (DCCSV) was carried out at accumulation potential $E_{acc} = -200$ mV and accumulation time $t_{acc} = 60$ s whilst stirring. Differential pulse anodic stripping voltammetry (DPASV) was carried out with optimum accumulation parameters: $E_{acc} = -1000$ mV and $t_{acc} = 15$ s.

EVLS was used for elucidation of the electrochemical processes. This technique is based on elimination of selected contributions from the total registered current under assumption that the total current is the sum of partial currents (Eq. (2)) [33–38]. These partial currents include the charging current (I_c), the diffusion controlled current (I_d), the kinetic current (I_k) and the so called irreversible current (I_{ir}).

$$I = \Sigma I_j = I_k + I_c + I_d + I_{ir} \quad (2)$$

According to the theory of EVLS, each particular current can be

expressed by Eq. (3),

$$I_j = W_j(v)\ Y_j(E) = vxY_j(E) \quad (3)$$

where I_j represents a particular current, $W_j(v)$ represents a function of the scan rate, $Y_j(E)$ is a function of the potential, and v is the scan rate [33,38,39]. This basic postulate of EVLS is not valid in the case of more complicated processes, e.g., diffusion-controlled reduction/oxidation in adsorbed state or diffusion-controlled reduction/oxidation preceded by a kinetically controlled reaction [35,37,38,40–43].

Statistical and validation parameters were calculated using software QC-Expert 3.3 (TriloByte Statistical Software, Czech Republic). *LOD*s and *LOQ*s, respectively, were calculated using direct signal method according to IUPAC [44,45]. The confidence (newly according to IUPAC the term "coverage" should be used) intervals were calculated on the level of significance $\alpha = 0.05$ [45].

3. Results and discussion

3.1. Voltammetric behavior of DMPS

3.1.1. pH dependence

The influence of pH on voltammetric behavior of DMPS was investigated by DCCSV in Britton-Robinson buffer (BRB). For pH 1 0.1 M HCl and for pH 13 0.1 M NaOH were used. Prior to each measurement, the electrode surface was pre-treated by 5 CV scans in the range from E_{in} −200 mV to E_{fin} −1800 mV in order to clean the electrode surface. After the pretreatment step, DMPS was accumulated at $E_{acc} = 0$ mV for 60 s whilst stirring.

One prominent DMPS peak was observed in a wide range of pH at both used electrodes (i.e., m-AgSAE and p-AgSAE) (Fig. 2) which apparently consisted of two merged peaks corresponding to reduction of two thiol moieties. The double-peak/shoulder shape is clearly visible on p-AgSAE at pH 4–7.

With increasing pH, peak potential was shifted towards more negative potentials (slope −59 mV/pH at p-AgSAE (Fig. 2A, Eq. (4)) and −51 mV/pH at m-AgSAE (Fig. 2B, Eq. (5)), confirming a proton-coupled electron transfer corresponding to the reduction of thiol bond, with Nernstian behavior up to pH 10 (Eq. (5)) reflecting DMPS acid dissociation constants ($pK_{a\ (sulfonic)} = 1.1$; $pK_{a\ 2,3\ (thiols)} = 9.4$, 12.1 [32,46,47]).

At p-AgSAE, DMPS signal at pH 12 and 13 was poorly developed and composed of several wider overlapping peaks. Furthermore, handling of DMPS solutions under strongly alkaline conditions is problematic due to oxidation of thiol groups when exposed to oxygen, as described in Ref. [48,49].

$$E_p\ [mV] = -(59.54 \pm 0.98)[mV]\ pH + (-121.4 \pm 7.8)\ [mV]$$
$$r = 0.998 \quad (4)$$

$$E_p\ [mV] = -(50.9 \pm 2.8)[mV]\ pH + (-156 \pm 22)\ [mV]\ r = 0.983 \quad (5)$$

The highest and best developed peak was obtained at pH 10 for p-AgSAE and pH 5 for m-AgSAE and these values were chosen as optimal for further experiments.

3.1.2. Cleaning/activation procedure

Due to strong chemisorption of thiol groups on p-AgSAE surface and consequent electrode fouling, suitable cleaning/activation procedure had to be optimized to obtain repeatable signals. Without any cleaning step, the peak currents decreased to less than 50% of the original height after seven consecutive voltammetric scans, with significant shift of the peak potential towards more negative values. Mechanical polishing of the electrode surface

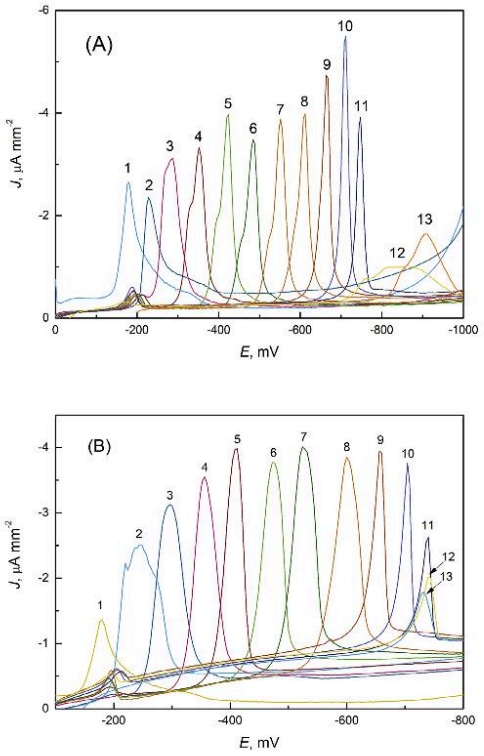

Fig. 2. DCCS voltammograms of 100 μmol L^{-1} DMPS at p-AgSAE (A) and m-AgSAE (B) in BRB of pH 2–12, in 0.1 M NaOH pH 13 and 0.1 M HCl pH 1. $E_{in} = 0$ mV, $E_{fin} = -1000$ mV (A), $E_{in} = -100$ mV, $E_{fin} = -800$ mV (B), $E_{acc} = 0$ mV, $t_{acc} = 60$ s, $v = 100$ mV s^{-1}. Numbers above voltammograms correspond to pH.

before each experiment resulted in poor repeatability of peak currents ($RSD = 19.6\%$, $N = 10$). CV proved to be the most suitable cleaning technique to obtain reproducible voltammograms. Therefore, the electrode was cleaned/activated in the sample solution by ten consecutive CV scans from −200 mV to −1800 mV at scan rate 500 mV s^{-1} before DCCSV run. Although the peak currents were slightly lower than those obtained using mechanical cleaning only, repeatability of the signal improved significantly. Slight shift of the peak potential towards more negative values was still observed at p-AgSAE, possibly corresponding to the electrode corrosion caused by dissolution of adsorbed metal-DMPS complex into buffer solution. Furthermore, the use of different regeneration potentials was tested in the range from +200 mV to −200 mV and from −1600 mV to −1800 mV, respectively, with no significant improvements compared to simpler electrode activation by CV only.

The fouling effect was less prominent at m-AgSAE. Therefore, sufficient cleaning procedure consisted of three consecutive CV scans at $v = 500$ mV s^{-1} in the range from 0 mV to −1000 mV.

3.1.3. Influence of accumulation potential and time

The accumulation potentials from 100 to −400 mV were tested for p-AgSAE. The highest peaks were obtained at accumulation

Table 1
Effect of applied accumulation potential (E_{acc}) on the 100 μM DMPS peak current density (J_p) and its repeatability (expressed as relative standard deviation (*RSD*)). Recorded on p-AgSAE and on m-AgSAE using DCCSV, t_{acc} = 60 s, $E_{in} = E_{acc}$, $E_{fin} = -800$ mV, v = 100 mV s^{-1}. BRB pH 10 was used for p-AgSAE, pH 5 was used for m-AgSAE.

p-AgSAE			m-AgSAE		
E_{acc}, mV	$-J_p$, μA mm^{-2}	RSD, %	E_{acc}, mV	$-J_p$, μA mm^{-2}	RSD, %
100	1.12 ± 0.19	15.7	100	6.08 ± 0.29	4.8
0	1.24 ± 0.25	20.6	50	4.21 ± 0.17	4.1
−100	0.980 ± 0.033	3.3	0	5.591 ± 0.091	1.6
−200	0.820 ± 0.016	1.9	−50	4.51 ± 0.037	0.8
−300	0.653 ± 0.015	2.4	−100	4.330 ± 0.080	1.8
−400	0.550 ± 0.073	1.3	−200	1.76 ± 0.12	6.8

potentials from 100 mV to 0 mV, but their repeatability was poor (*RSD* higher than 15%, $N = 3$) (Table 1). Due to very good repeatability and sufficient peak high, the accumulation potential of −200 mV was chosen as optimum.

At m-AgSAE, the accumulation potentials from 100 to −200 mV were tested (Table 1). The highest peaks were obtained at E_{acc} from 100 mV to 0 mV. On the other hand, only negligible peaks were observed at potentials lower than −200 mV. Therefore, the E_{acc} = 0 mV was chosen as optimum.

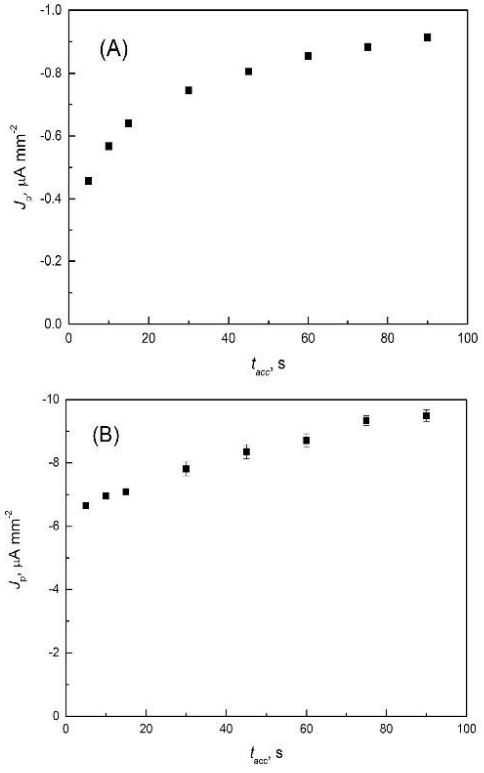

Fig. 3. Dependence of 100 μM DMPS peak height recalculated to corresponding current density on the time of accumulation (t_{acc}) at p-AgSAE (A) and at m-AgSAE (B) using DCCSV. Conditions used for p-AgSAE were BRB of pH 10 and E_{acc} = −200 mV, for m-AgSAE BRB of pH 5 and E_{acc} = 0 mV. Scan rate v = 100 mV s^{-1}, $E_{in} = E_{acc}$, E_{fin} = −800 mV. The confidence intervals were calculated on the level of significance α = 0.05.

Accumulation times from 5 to 90 s were examined at p-AgSAE at E_{acc} = −200 mV. The peak current increased with increasing accumulation time resulting in the dependence in the shape of an adsorption isotherm (Fig. 3A). Application of the accumulation time above 90 s has led to lower repeatability. The optimum t_{acc} was set to 30 s, as its additional prolongation increases the peak height only marginally.

Similarly, t_{acc} from 5 s to 90 s were examined at m-AgSAE with E_{acc} = 0 mV. The DMPS peak increased with increasing time. The dependence was approximately linear, contrary to the tendency shown in previous experiment with p-AgSAE. The accumulation time of 15 s was chosen as optimum, because of sufficiently high peak and excellent repeatability.

3.1.4. DPCSV concentration dependences

Concentration dependences of DMPS in the range from 0.1 to 10 μmol L^{-1} at p-AgSAE and m-AgSAE were measured under previously established optimum conditions summarized in Table 2 using DPCSV (Fig. 4, Fig. 5).

At p-AgSAE, the dependence of DPCSV peak height on DMPS concentration is linear from 0.3 μmol L^{-1} to 2.0 μmol L^{-1}. In the whole tested concentration range, the dependence has logarithmic shape (Eq. 6) corresponding to an adsorption isotherm of the accumulation process. Furthermore, a notable shift of the peak potential towards more negative values with increasing concentration of DMPS was observed. This reflects complexity of DMPS adsorption on p-AgSAE surface and of its consequent desorption, with regard to the electrode history, fouling, corrosion, and confirms that strength of metal-thiol bond is influenced by the surface composition, structure, and morphology at atomic level. *LOD* = 0.26 μmol L^{-1} and *LOQ* = 0.79 μmol L^{-1} were obtained.

m-AgSAE seems to be more suitable for this application, since the peak potentials were more stable (Table 1). Concentration dependency of DMPS at m-AgSAE was approximately linear in both tested concentration ranges, i.e., from 0.1 μmol L^{-1} to 1 μmol L^{-1} and from 1 to 10 μmol L^{-1}. Reached *LOD* and *LOQ* were 0.1 μmol L^{-1} and 0.3 μmol L^{-1}, respectively. The differences in performance between the two electrodes are in good agreement with previously published studies.

3.2. DPCSV determination of DMPS in model samples

The DPCSV with above optimized parameters and calibration curve method was used to determine DMPS in model samples of drugs and of urine. Validation parameters calculated from 5 repeated determinations during one day are shown in Table 3. The found content of DMPS in the drug Dimaval at p-AgSAE was lower than the declared one. On the contrary, the declared contents of DMPS were included in confidence intervals of results obtained using m-AgSAE. *RSD* was below 2.6% and 1.5% for p-AgSAE and m-

Table 2
Optimum conditions and statistical parameters of DMPS determinations using DPCSV at p-AgSAE and m-AgSAE; r – correlation coefficient.

Working electrode	pH	E_{acc}, mV	t_{acc}, s	Concentration range, μmol L^{-1}	Slope, mA mm^{-2} mol^{-1} L	Intercept, μmol L^{-1}	r	*LOD*, μmol L^{-1}	*LOQ*, μmol L^{-1}
p-AgSAE	10	−200	30	2.0–10.0	logarithmic (Eq. 6)	logarithmic (Eq. 6)	0.999	–	–
				0.3–2.0	–(33.7 ± 1.4)	0.27 ± 0.95*	0.997	0.26	0.79
m-AgSAE	5	0	15	1.0–10.0	–(29.2 ± 9.3)	18.4 ± 5.7	0.988	–	–
				0.1–1.0	–(83.3 ± 6.4)	7.2 ± 3.7	0.998	0.1	0.3

* Insignificant on the level of significance $\alpha = 0.05$.
c[μmol L^{-1}] = −(42.4 ± 1.9) ln(J_p[nA mm^{-2}] + (−0.560 ± 0.069))[μmol L^{-1} nA^{-1} mm^2] + (−64.5 ± 3.9)[μmol L^{-1}] r = 0.999 (Eq. 6).

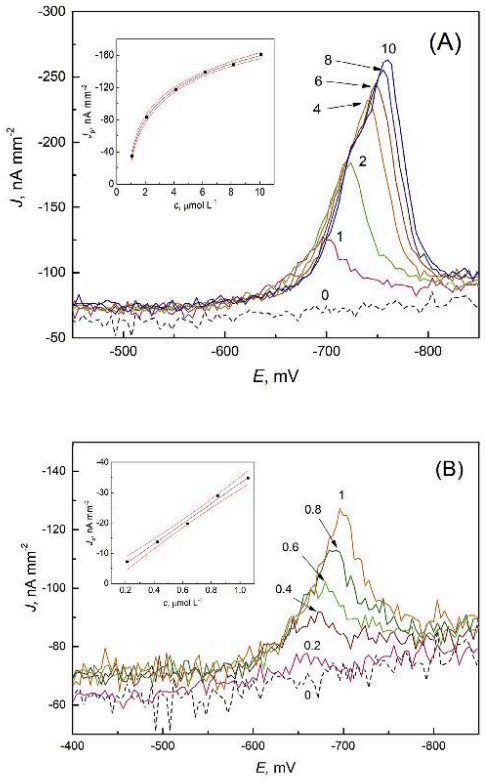

Fig. 4. DPCS voltammograms of DMPS at p-AgSAE in the concentration range A) from 1 μmol L^{-1} to 10 μmol L^{-1} and B) from 0.2 μmol L^{-1} to 1 μmol L^{-1} in BRB of pH 10. E_{acc} = −200 mV, t_{acc} = 30 s, ν = 100 mV s^{-1}, E_{in} = E_{acc}. Insets: DMPS calibration concentration dependences based on peak current density A) from 1 μmol L^{-1} to 10 μmol L^{-1} and B) from 0.2 μmol L^{-1} to 1 μmol L^{-1}.

AgSAE, respectively.

In urine model samples, however, the repeatability at p-AgSAE was significantly worse. The DMPS peak diminished with every consecutive measurement. It could be explained probably by fouling effects caused by urine components. This demonstrates the complexity of voltammetric analysis of biological matrices in combination with the contribution of a solid electrode history. The found DMPS amounts were in good agreement with declared contents at both electrodes. However, the m-AgSAE yielded with adequate precision more satisfactory *RSD*s of 5.2% and 3.0% for 1.0 μmol L^{-1} and 10 μmol L^{-1} sample, respectively.

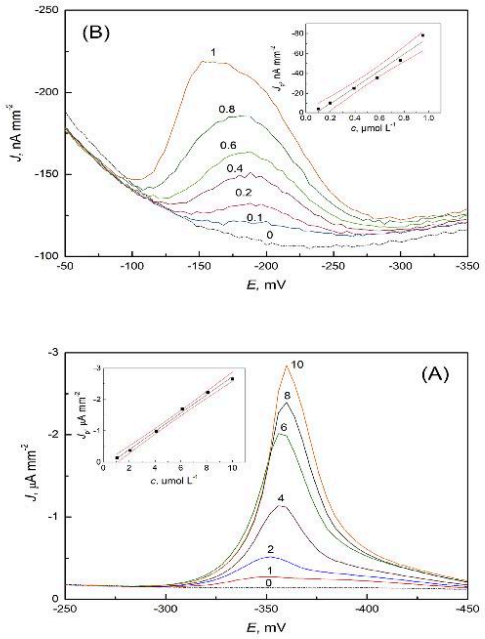

Fig. 5. DPCS voltammograms of DMPS at m-AgSAE in the concentration range A) from 1 μmol L^{-1} to 10 μmol L^{-1} and B) from 0.1 μmol L^{-1} to 1 μmol L^{-1} in BRB of pH 5. E_{acc} = 0 mV, t_{acc} = 15 s, ν = 100 mV s^{-1}, E_{in} = E_{acc}. Insets: DMPS calibration concentration based on peak current density A) from 1 μmol L^{-1} to 10 μmol L^{-1} and B) from 0.1 μmol L^{-1} to 1 μmol L^{-1}.

3.3. Elimination voltammetry with linear scan (EVLS)

The dependence of the DMPS cathodic peak height on the scan rate was investigated using DCV at both electrodes. Scan rates from 10 to 640 mV s^{-1} have been applied. The peak current density was directly proportional to the scan rate, confirming that the process is adsorption-controlled, as shown in Eq. (7) (p-AgSAE) and Eq. (8) (m-AgSAE).

$$J_p\ [(\text{nA mm}^{-2}] = -(7.340 \pm 0.098)\ [\text{nA mm}^{-2}\ \text{mV}^{-1}\ \text{s}]\nu\ [\text{mV s}^{-1}\ ] + (-125 \pm 27)\ [\text{nA mm}^{-2}];\ r = 0.999 \quad (7)$$

$$J_p\ [(\text{nA mm}^{-2}] = -(68.7 \pm 2.8)\ [(\text{nA mm}^{-2}\ \text{mV}^{-1}\ \text{s}]\nu\ (\text{mV s}^{-1}) + (-990 \pm 780)\ [\text{nA mm}^{-2}];\ r = 0.995 \quad (8)$$

The elimination curves have been calculated according to equations (S2..S8) in supplement of ref. [41] (where the most

Table 3
Accuracy of five repeated DPCSV determinations of DMPS at m-AgSAE and p-AgSAE in model samples. Conditions are the same as in Table 2.

	DMPS added, μmol L^{-1}	DMPS found, μmol L^{-1}	Recovery, %	RSD, %	Bias, %
Dimaval					
p-AgSAE	1.0	0.940 ± 0.021	94.0 ± 2.1	2.6	−6.4
	10.0	9.77 ± 0.16	97.7 ± 1.5	1.8	−2.3
m-AgSAE	1.0	1.020 ± 0.013	102.0 ± 1.3	1.5	2.0
	10.0	10.190 ± 0.071	101.90 ± 0.71	0.8	1.9
Urine					
p-AgSAE	1.0	1.072 ± 0.073	107.2 ± 7.3	7.8	6.7
	10.0	9.2 ± 1.0	92 ± 10	12.6	−9.1
m-AgSAE	1.0	0.980 ± 0.045	97.7 ± 4.5	5.2	−2.3
	10.0	10.27 ± 0.27	102.7 ± 2.7	3.0	3.6

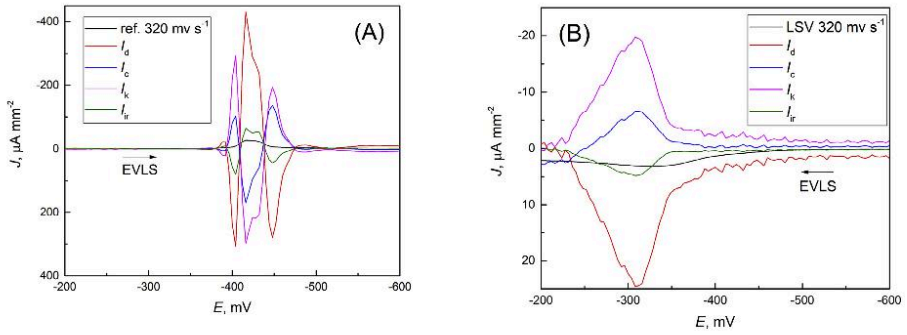

Fig. 6. DC voltammograms (black) and elimination voltammograms of 0.2 mmol L^{-1} DMPS at m-AgSAE in BRB pH 5. Scan rates used for calculation of the depicted elimination curves: 80 mV s^{-1}, 160 mV s^{-1}, 320 mV s^{-1}, and 640 mV s^{-1}, reference scan rate curve ν = 320 mV s^{-1}. Cathodic (A) and anodic (B) scans are depicted. Elimination curves correspond to diffusion (I_d), charging (I_c), kinetic (I_k), and irreversible (I_{ir}) currents.

important information on EVLS is summarized). The controlling processes revealed by ELSV were analogous on both tested electrodes.

Scan rates from 10 to 1280 mV s^{-1} were applied using optimum parameters on both tested electrodes. Two sets of scan rates with the highest signal to noise ratios were evaluated at m-AgSAE a) 10 mV s^{-1}, 20 mV s^{-1}, 40 mV s^{-1} and 80 mV s^{-1} and b) 80 mV s^{-1}, 160 mV s^{-1}, 320 mV s^{-1}, and 640 mV s^{-1} for both cathodic and anodic scans. Similarly, two sets of scan rates were evaluated at p-AgSAE: a) 10 mV s^{-1}, 20 mV s^{-1}, 40 mV s^{-1} and 80 mV s^{-1} and b) 160 mV s^{-1}, 320 mV s^{-1}, 640 mV s^{-1}, and 1280 mV s^{-1} for both cathodic and anodic scans.

At m-AgSAE, the cathodic peak was separated by ELVS into two peaks at about −415 mV and −440 mV, respectively, corresponding

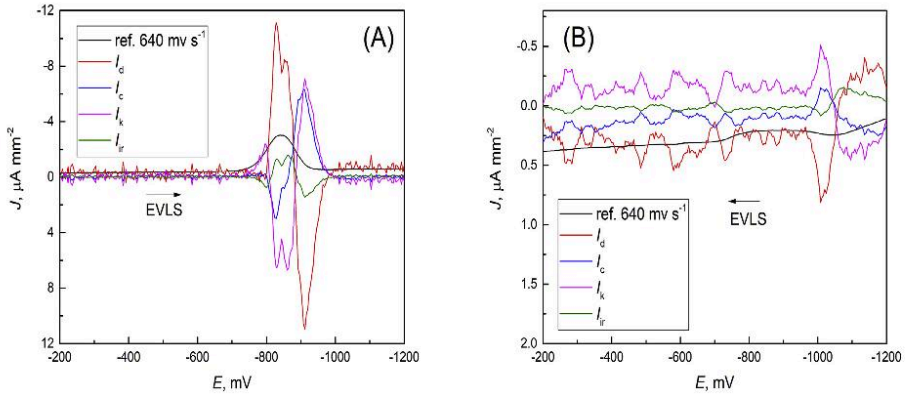

Fig. 7. DC voltammograms (black) and elimination voltammograms of 0.2 mmol L^{-1} DMPS at p-AgSAE in BRB pH 10. Scan rates used for calculation of the depicted elimination curves: 160 mV s^{-1}, 320 mV s^{-1}, 640 mV s^{-1}, and 1280 mV s^{-1}, reference scan rate curve ν = 640 mV s^{-1}. Cathodic (A) and anodic (B) scans are depicted. Elimination curves correspond to diffusion (I_d), charging (I_c), kinetic (I_k), and irreversible (I_{ir}) currents.

to two consecutive reductions in adsorbed state. At the scan rate set from 80 mV s^{-1} to 640 mV s^{-1}, these reductions have been preceded by a kinetically controlled process at about −400 mV (Fig. 6A).

A single anodic counter peak at about −390 mV corresponded to diffusion process with preceding kinetic process, i.e., to oxidation of mercury surface of m-AgSAE in the presence of DMPS, products of which have been transported by diffusion from the bulk solution (Fig. 6B).

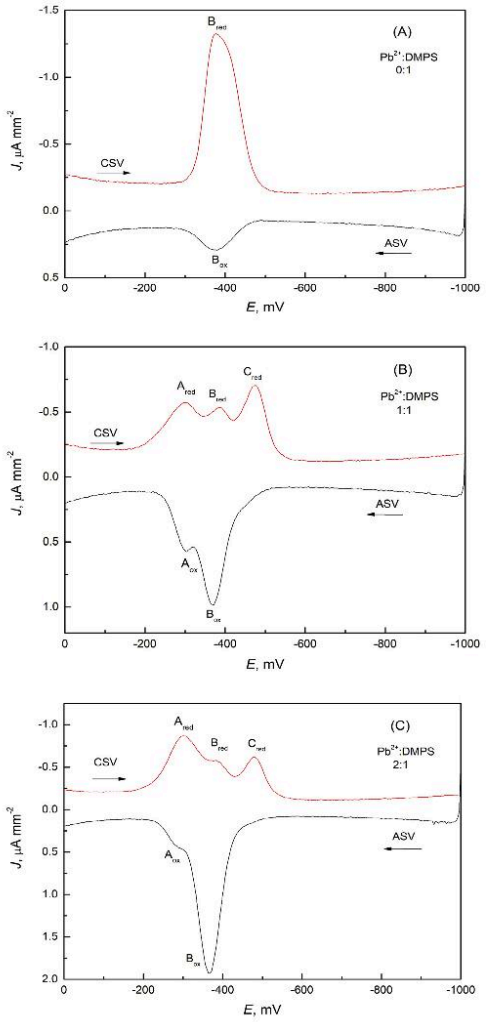

Fig. 8. DP CS and DP AS voltammograms of 10 µmol L^{-1} DMPS in acetate buffer pH 5 corresponding to [Pb^{2+}: DMPS] ratios of 0:1(A), 1:1(B), and 2:1(C). Red curve corresponds to the cathodic scan, E_{acc} = 0 mV, t_{acc} = 15 s. Black curve corresponds to reverse anodic scan with t_{acc} = 15 s at E_{acc} = −1000 mV, v = 20 mV s^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Similarly, at the p-AgSAE, two consecutive reductions in adsorbed state were identified at about −790 mV and −830 mV, respectively (Fig. 7A). In anodic scans, a poorly developed peak at about −710 mV, corresponding to a diffusion-controlled process, is observed only at scan rates higher than 160 mV s^{-1}. No significant process was revealed in anodic scan (Fig. 7B).

Obtained ELSV results were in good agreement with the other experimentally obtained data. The only significant difference was observed in peak potentials between p-AgSAE and m-AgSAE. However, please note that these experiments were carried out under previously optimized conditions that are different for each electrode, most notably pH.

3.4. Voltammetric behavior of DMPS in the presence of Pb^{2+}

From the electrochemical point of view, DMPS in the presence of Pb^{2+} ions is complicated system with multiple possible processes, including Pb(DMPS) formation in bulk solution, Hg(DMPS) formation by chemisorption on the electrode surface, Pb/Hg transmetalation reactions, reduction of free Pb^{2+} ions, and reverse reactions. Voltammetric titrations of DMPS by $Pb(NO_3)_2$, investigated by DPASV an DCCSV were used for the investigation of electrode processes. Prior to titration experiments, both DPV (Eq. 9) and DPASV (Eq. (10)) dependencies of peak current densities on Pb^{2+} ion concentration at m-AgSAE were established in the range from 0.1 to 100 µmol L^{-1}.

$$j_p\ [\text{nA mm}^{-2}] = -(2.56 \pm 0.05)\ [\text{nA mm}^{-2}\ \mu\text{mol}^{-1}\ \text{L}]\ c\ [\mu\text{mol L}^{-1}] + (-19.7 \pm 2.5)\ [\text{nA mm}^{-2}]\ r = 0.997 \quad (9)$$

$$j_p\ [\text{nA mm}^{-2}] = (111.0 \pm 7.6)\ [\text{nA mm}^{-2}\ \mu\text{mol}^{-1}\ \text{L}]\ c\ [\mu\text{mol L}^{-1}] + (184 \pm 100)\ [\text{nA mm}^{-2}]\ r = 0.995 \quad (10)$$

Voltammetric titrations were carried out by consecutive additions of 1 mmol L^{-1} of $Pb(NO_3)_2$ into 100 µmol L^{-1} DMPS solution in acetate buffer of pH 5.0. DPAS and DPCS voltammograms for the Pb^{2+}: DMPS ratio of 0:1, 1:1, and 2:1 are shown in (Fig. 8A–C).

The occurring reactions are as follows:

With no added Pb^{2+} (Fig. 8 A), Hg(DMPS) is formed on the electrode surface during the accumulation step and subsequently reduced during cathodic scan with single well-developed peak at −380 mV (B_{red}). A less pronounced counter-peak appears during the DPASV anodic scan, corresponding to the reverse process of Hg^0

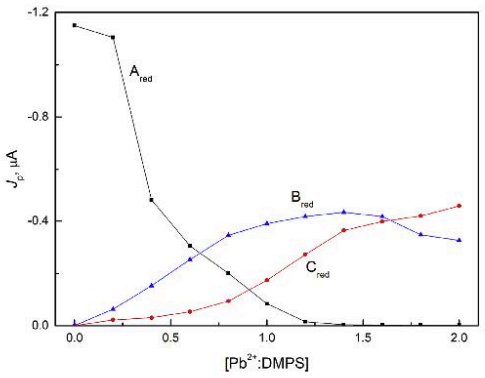

Fig. 9. Dependency of DPCSV peak current densities on [Pb^{2+}:DMPS] ratios 0:1 to 2:1. Conditions are the same as in Fig. 8.

Table 4
Comparison of voltammetric methods for DMPS determination.

Method	Working electrode	LDR, μmol L^{-1}	*LOQ*, μmol L^{-1}	*LOD*, μmol L^{-1}	Ref.
LSV	glassy-carbon electrode modified with multi-walled carbon nanotubes	18–140 260–690	41	14	[21]
DPCSV	p-AgSAE	0.3–2.0	0.8	0.3	this work
DPCSV	m-AgSAE	1.0–10.0 0.1–1.0	– 0.3	– 0.1	this work

LDR – linear dynamic range.

oxidation to form a Hg(DMPS) complex (B_{ox}).

B_{red}: Hg(DMPS) → Hg^0 + DMPS (adsorption controlled)

B_{ox}: DMPS + Hg^0 → Hg(DMPS)

At Pb^{2+}:DMPS ratio 1:1, further two cathodic peaks have been registered (Fig. 8 B). Peak at about −300 mV (A_{red}) corresponds to transmetalation reaction of the Hg(DMPS) with the Pb^{2+} to form a Pb-DMPS complex. The addition of Pb^{2+} causes decrease of B_{red} peak due to Pb^{2+}-DMPS formation in the solution. Reduction peak of Pb^{2+} is also present at the same potential. The reduced lead is accumulated at the electrode surface as lead amalgam Pb(Hg). The Pb-DMPS complex is reduced at about −500 mV to Pb^0(Hg). Two peaks, A_{ox} and B_{ox} appear at AS voltammogram, corresponding to the reverse processes of A_{red} and B_{red}. The C_{ox} peak is not present, because there is effectively no free DMPS in the solution.

A_{red}: Hg(DMPS) + Pb^{2+} + $2e^-$ → Pb(DMPS) + Hg^0

A_{ox}: Pb(DMPS) + Hg^0 → Hg(DMPS) + Pb^{2+} + $2e^-$ (adsorption controlled)

B_{red}: Hg(DMPS) + $2e^-$ → Hg^0 + DMPS

Pb^{2+} + $2e^-$ + (Hg) → Pb^0(Hg)

B_{ox}: Pb^0(Hg) → Pb^{2+} + $2e^-$ + (Hg)

C_{red}: Pb(DMPS) + $2e^-$ + (Hg) → Pb^0(Hg) + DMPS

At Pb^{2+}:DMPS ratio 2:1, there is excess of Pb^{2+} ions and no free DMPS in the solution (Fig. 8C). The number of observed cathodic and anodic peaks remains the same as in the above-described voltammograms. As a consequence of Pb^{2+} ions excess, A_{red} peak increased, while B_{red} and C_{red} remained practically unchanged. Increase of B_{ox} peak is caused by the presence of free Pb^{2+} ions which are available for deposition during DPASV accumulation step at $E_{acc} = -1000$ mV.

A_{red}: Hg(DMPS) + Pb^{2+} + $2e^-$ → Pb(DMPS) + Hg^0

A_{ox}: Pb(DMPS) + Hg^0 → Hg(DMPS) + Pb^{2+} + $2e^-$

B_{red}: Pb^{2+} + $2e^-$ + (Hg) → Pb^0(Hg)

B_{ox}: Pb^0(Hg) → Pb^{2+} + $2e^-$ + (Hg) (adsorption controlled)

C_{red}: Pb(DMPS) + $2e^-$ + (Hg) → Pb^0(Hg) + DMPS

The complete voltammetric titration curves of both DPCSV and DPASV confirm the outlined mechanism. Furthermore, they demonstrate that the prevalent Hg(DMPS) complex at the pH of 5.0 has a 1:1 ratio of ligand and Hg (Fig. 9). Formation of $Hg(DMPS)_2$ complex [18] was not observed, possibly due to more complicated behavior of DMPS in the presence of Pb^{2+} on amalgam electrode than on a bismuth one. Finally, possibility of Pb^{2+} determination alongside DMPS by stripping voltammetry can be considered. It can be used for the determination of total Pb^{2+} concentration, both free and bound to a complex.

4. Conclusions

A new method for the determination of DMPS was developed with the use of DPCSV at two silver solid amalgam electrodes (p-AgSAE and m-AgSAE). The method was successfully applied to model samples of drug Dimaval and human urine spiked with DMPS with *LOD*s 0.1 μmol L^{-1} and 0.26 μmol L^{-1} at m-AgSAE and p-AgSAE, respectively. These values are two orders of magnitude lower compared to the only previously reported voltammetric method based on then use of carbon nanotubes modified electrode [21] (Table 4). m-AgSAE gave more stable response and lower background current than p-AgSAE. This effect was even more pronounced in the case of urine analysis.

Furthermore, various electrochemical methods have been employed to elucidate the undergoing processes. CV and EVLS were used for the elucidation of controlling processes, confirming two consecutive reductions in adsorbed state with proton/electron coupled transport. In addition, voltammetric titrations of DMPS by Pb^{2+} ions were used to assign undergoing reactions to observed peaks. The most prominent complex at pH 5 exhibits Pb to DMPS the ratio of 1:1. It can be envisaged that with the use of advanced statistical methods such as multivariate complex analysis, the proposed method could be used for simultaneous determination of Pb^{2+} and DMPS in human urine for clinical purposes.

CRediT authorship contribution statement

Vojtěch Hrdlička: Formal analysis, Writing - review & editing. **Marta Choińska:** Investigation, Writing - original draft. **Beatriz Ruiz Redondo:** Investigation. **Jiří Barek:** Supervision. **Tomáš Navrátil:** Conceptualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Publication 3 – Submitted for publication

Differential pulse voltammetric determination of homovanillic acid as a tumor biomarker in human urine after hollow fiber-based liquid-phase microextraction

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ABSTRACT

Novel method for the determination of a tumor marker homovanillic acid (HVA) in human urine was developed. Combination of hollow fiber – based liquid-phase microextraction (HF-LPME) and differential pulse voltammetry (DPV) at a cathodically pre-treated boron doped diamond electrode (BDDE) has been applied for these purposes. Optimum conditions were: butyl benzoate as supported liquid membrane (SLM) formed on polypropylene HF, 0.1 mol L^{-1} HCl as donor phase, 0.1 mol L^{-1} sodium phosphate buffer of pH 6 as acceptor phase, and 30 min extraction time. HF-LPME-DPV concentration dependence was linear in the range from 0.25 to 100 μmol L^{-1}. Limits of quantification (*LOQ*) and detection (*LOD*) were 1.2 and 0.4 μmol L^{-1}, respectively. The applicability of the developed method was verified by analysis of human urine. Standard addition method was used, found HVA concentration was 13.5 ± 1.3 μmol L^{-1}, *RSD* = 9.3 % (n=5).

Keywords

Liquid phase microextraction

Homovanillic acid

Hollow fiber – based liquid-phase microextraction

Differential pulse voltammetry

Catecholamine metabolites

Neuroblastoma biomarkers

Boron doped diamond electrode

List of abbreviations

DPV differential pulse voltammetry

E_{act} activation potential

EF enrichment factor

HF hollow fiber

HF-LPME hollow fiber liquid phase microextraction

HVA homovanillic acid

LOD limit of detection

LOQ limit of quantification

PBS phosphate buffer saline

RSD relative standard deviation

SLM supported liquid membrane

t_{act} activation time

t_{ext} extraction time

V_{AS} volume of acceptor solution

V_{DS} volume of donor solution

1. Introduction

Since 1970s, homovanillic acid (HVA) (Fig. 1) has been used as a biomarker of various diseases, as well as a target molecule for measuring overall dopamine activity in human body [1-3]. Increase of HVA concentration in body fluids is linked to metabolic disorders[4, 5], cancerous diseases of adrenal medulla pheochromocytoma [6] and neuroblastoma [7] as well as neurological disorders such as autism [8] and posttraumatic stress disorder [9], whilst

decreased HVA concentration is typical for depression [10] and Parkinson´s disease [1]. Determination of HVA concentration in plasma, urine or cerebrospinal fluid is relevant for diagnosis [11], monitoring of disease progression [12, 13], and drug dosage optimization [14]. Screening tests for neuroblastoma in infants based on determination of urinary HVA and other catecholamine metabolites have been successfully employed as a way to decrease mortality by early diagnosis [15-17]. Reported molar concentration of urinary HVA in healthy 6-month-old infants is 34.6±7.6 μmol L^{-1} [18, 19], whilst the cut-off concentration increase for neuroblastoma is 2.5 of standard deviation [20], hence 53.6 μmol L^{-1}. These values are approximate, as reference concentrations in different studies tend to vary [18, 21, 22]. In addition to being strongly influenced by dietary flavonoids [23], urinary HVA levels are also age-dependent. Sampling strategies such as collection of urine over 24 hours or correction of HVA concentration to creatinine excretion are discussed in dedicated publications [3, 24-26].

Numerous analytical methods for the determination of HVA in body fluids are based on modern separation techniques such as liquid chromatography [27-30], gas chromatography [8, 23], and capillary electrophoresis [21, 31]. However, electrochemical methods present a viable alternative as a way to decrease costs of the used instrumental equipment, chemicals, and decrease overall environmental impact of mass screenings.

Due to the presence of aromatic phenolic group, HVA is electrochemically oxidisable applying various types of electrode materials [32-36]. Using amperometry or pulse voltammetry, HVA can be determined in wide concentration ranges with sub-micromolar detection limits [37, 38]. Disadvantage of these methods is often their limited use in complex biological matrices due to the presence of multiple electrochemically active interferences or high-molecular compounds that can adsorb on the electrode surface resulting in its fouling [39]. In order to minimize these undesirable effects, electroanalysis can be complemented with a suitable membrane separation technique, as it was previously shown in our study on the hollow fiber – based liquid-phase microextraction (HF-LPME) and differential pulse voltammetric (DPV) determination of urinary vanillylmandelic acid (VMA)[40] structurally related to HVA.

HF-LPME is a novel technique for accelerated pre-treatment (preliminary separation) of samples from complex matrices, using disposable porous plastic HFs [41-43]. Three phase HF-LPME of acidic compounds involves transport of the analyte of interest from acidic donor solution to supported liquid membrane (SLM) realized by a suitable liquid in the hollow fiber pores, and subsequently into basic acceptor solution inside the fiber lumen [44]. Extraction

efficiency and selectivity is greatly influenced by selection of the most suitable solvent for SLM formation and pH of donor as well as acceptor solution, with regards to acid-base constants and polarity of the analyte [45]. Aqueous acceptor solution is also suitable for immediate voltammetric analysis without need for additional treatment, including possibility of direct analysis inside the separation fiber [46-48]. Detailed theory and application of HF-LPME is discussed in recent reviews [49-51].

This study deals with the development of new, fast, and simple method for determination of urinary HVA and its optimization for an application in neuroblastoma screenings based on HF-LPME with subsequent DPV determination on boron doped diamond electrode (BDDE).

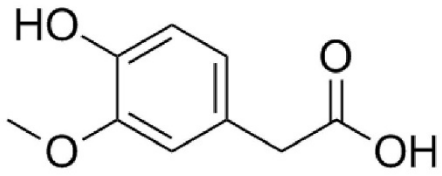


Fig. 1. Structure of HVA.

2. Experimental

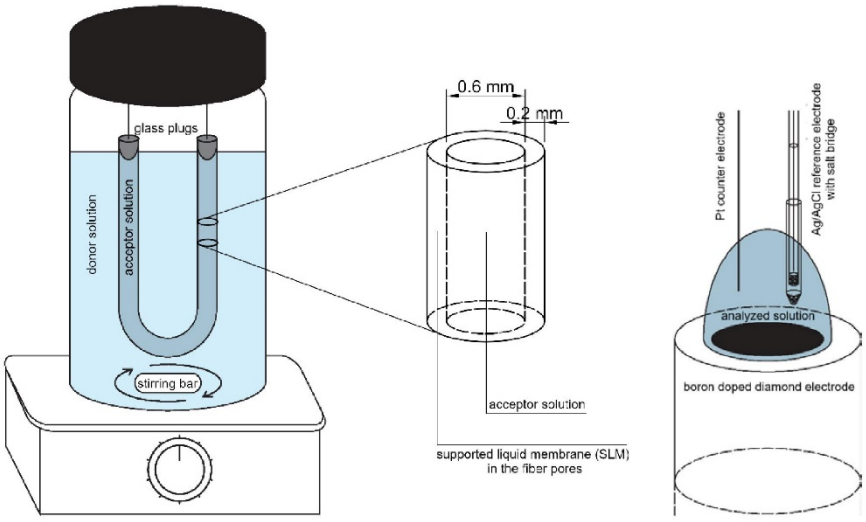
2.1 Material

HVA (4-Hydroxy-3-methoxyphenylacetic acid, CAS number: 306-08-1, Sigma Aldrich, Czech Republic) stock solution ($c = 1 \cdot 10^{-3}$ mol L^{-1}) was prepared by dissolving 182.2 mg of the substance in 1.0 L of deionized water (conductivity < 0.05 μS cm^{-1}, Millipore Mili plus Q system, USA). It was kept in cold and dark. Sodium chloride, acetone, sodium hydroxide, and hydrochloric acid (35%) (all p.a.) were purchased from Ing. Petr Švec - PENTA, Czech Republic. Sigma Aldrich, Czech Republic, was the supplier of the following chemicals: pentyl benzoate (CPR grade), dihexyl ether (97%, p.a.), isoamyl benzoate (≥98%, food grade), hexyl benzoate (food grade); propyl benzoate (99%, p.a.), butyl benzoate (99%, p.a.), 1-phenyldecane (98%, p.a.), and 1-octanol (99%, p.a.). Phosphate buffer saline (PBS) was prepared by mixing appropriate amounts of 0.2 mol L^{-1} NaOH (p.a.) with 0.1 mol L^{-1} phosphoric acid (p.a.). Its ionic strength of $I = 0.55$ mol L^{-1} was adjusted by NaCl (p.a.) addition. Polypropylene HF Q3/2 Accurel (pore diameter 0.2 μm, wall thickness of 200 μm, and inner diameter of 600 μm.) was supplied by 3M Membrana (Germany).

2.2 Apparatus

PalmSens4 (PalmSens BV, Netherlands), controlled by a desktop computer equipped with Microsoft Windows 10 (64 bits) (Microsoft, USA) operating system and PSTrace version 5.6 (PalmSens BV, Netherlands) (electrochemical software) was used for voltammetric measurements. Three-electrode arrangement (see Fig. 2 right side) was used consisting of BDDE (Windsor Scientific, UK, diameter 3 mm) as the working electrode, Ag|AgCl| (3 mol·L^{-1} KCl) (type "RAE111" Monokrystaly, Czech Republic) as the reference electrode and platinum wire counter electrode (diameter 0.28 mm, Monokrystaly, Czech Republic). The working BDDE was positioned upside-down [52]. The reference electrode, to which all potentials are referred, was connected by a salt bridge ended with a frit (diameter 1.0 mm, S2) and filled with the same solution as acceptor phase used for the experiment. The apparatus and experimental arrangement are analogous as in our previous work [40].

Fig. 2. Scheme of the used HF-LPME extraction cell (left side) and of the used three electrode system for micro volume analysis (right side).



2.3 Hollow-fiber microextraction

20-mm long pieces of the used HFs were washed by sonication in acetone bath for 5 min, and left to dry on the air. To impregnate the pores of the HF, it was dipped into an organic solvent (time interval of 5 s was found to be optimal), air expelled from a syringe removed residual organic solvent from the fiber lumen. Thereafter, the prepared HF was filled with the

acceptor phase (approximately 10 μL). Glass plugs (laboratory made) served as seals on both HF ends. To perform the extraction (see Fig. 2 left side), the sealed HFs were completely immersed into the donor phase. This process was realized under continual stirring (1 500 rpm) with 2 cm long Teflon stir bar (P-LAB, Czech Republic) on the magnetic stirrer (AREC.X, Czech Republic). After the extraction was completed, the HF was immediately withdrawn from the donor solution, the plugs were removed, and the acceptor solution from the fiber lumen was transferred by a syringe to the surface of the BDDE (Fig. 2 right side) and the voltammetric scan was started.

Prior to the analyses, the urine samples were centrifuged for 5 min. at Eppendorf MiniSpin (Eppendorf, Czech Republic). If necessary, the centrifuged samples were stored in cold and dark. Number of repetitions of each experiment was five.

The coverage intervals were calculated on the significance level $\alpha = 0.05$. Detection limits (*LOD*s) and quantification limits (*LOQ*s) were calculated by direct method of analyte according to IUPAC [53].

3. Results and discussion

3.1 Direct DPV determination of HVA at BDDE

BDDE was chosen as suitable electrode material for voltammetric determination of HVA, as shown by Baluchova [38]. However, due to repulsion between HVA (which is present as an anion ($pK_{A,\ carboxylic} = 4.35$, $pK_{A,\ phenolic} = 10.34$) [29] in a neutral or alkaline hollow fiber extract) and negatively charged surface of O-terminated BDDE, anodic pre-treatment of BDDE was not suitable for this study. Such effect significantly shifted oxidation peak of HVA to the area of more positive potentials, caused broadening of the HVA peak and poor repeatability. Cathodic pre-treatment of BDDE at –1.0 V for 15 s was therefore employed to make the BDDE fully usable for HVA determination under the alkaline conditions. Pre-treatment potentials lower than –1.0 V have led to gradual increase of background current and to lower sensitivity. The end potential at pH 13 was set to +0.6 V to avoid oxidation of the electrode surface. It is worth noting that changing the BDDE surface termination from –O to –H is possible by application of highly negative potentials under acidic conditions [54], although it is a much slower process than vice-versa.

At cathodically pre-treated BDDE in 0.1 mol L^{-1} NaOH, HVA is oxidized in single well-developed peak at +0.38 V (Fig. 3A, B). Elimination voltammetry with linear scan [55-60], (EVLS) was utilized to elucidate the electrode processes (see Supporting Information) [61-

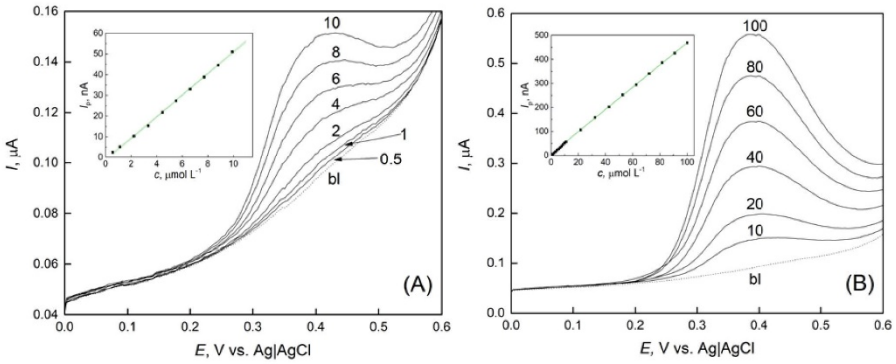
63]. Diffusion was found to be the controlling process of the HVA oxidation, with negligible contributions of kinetic, capacitance, and irreversible currents at all used scan rates (from 0.020 to 1.280 V s^{-1}). This is in agreement with mechanism proposed by Li [32] and later verified by Pfeifer [64].

Obtained DPV calibration dependence was linear from 0.5 to 100 µmol L^{-1} (Eq. 1). *LOQ* and *LOD* were 2.4 and 0.8 µmol L^{-1}, respectively.

I_p [nA] = (4.620 ± 0.021) c [µmol L^{-1}] + (6.6 ± 1.1) [nA] (R=0.9998) (Eq. 1)

Achieved *LOQ* and *LOD* are sufficient for the intended purpose of HVA determination in human urine. However, analysis of real urinary samples without a prior separation step is impossible, due to large amount of interfering electrochemically active compounds in urine.

Fig. 3. DP voltammograms of HVA (without LPME), sample volume 10 µL, supporting electrolyte: 0.1 mol·L^{-1} NaOH, working electrode: BDDE, concentration ranges of HVA: 0.5 − 10 µmol L^{-1} (A) and 10 − 100 µmol L^{-1} (B). Dotted curve: blank sample (bl). The numbers next to the curves correspond to HVA concentrations in µmol L^{-1}. Pre-treatment of BDDE: E_{act} = −1.0 V for t_{act} = 15 s. Inset: HVA DPV concentration dependence.



3.2 DPV determination of HVA at BDDE after HF-LPME

Main factors influencing the overall performance of HF-LPME were experimentally optimized to achieve maximum repeatability, sensitivity and selectivity towards HVA. These factors are: extraction time, compositions of organic liquid used in SLM, of donor solution and of acceptor solution. This strategy was already successfully used for the development of DPV determination of VMA after HF-LPME [40]. It was presumed that HVA can be extracted into basic acceptor solution inside the fiber lumen from acidic donor solution

through SLM in its neutral form. Donor solution volume was set to 10 mL in order to provide stable stirring and full immersion of HF used for extraction. Acceptor solution volume was 10 μL. Smaller acceptor solution volume would complicate manipulation with the extract and worsen the repeatability. On the other hand, increasing the acceptor solution volume leads to slight, yet undesired decrease of maximum possible extraction efficiency and pre-concentration factor. Stirring speed was set to 1500 rpm to ensure steady and robust transport of HVA from the bulk solution towards the fiber surface, without undesirable turbulent flow in the extraction cell.

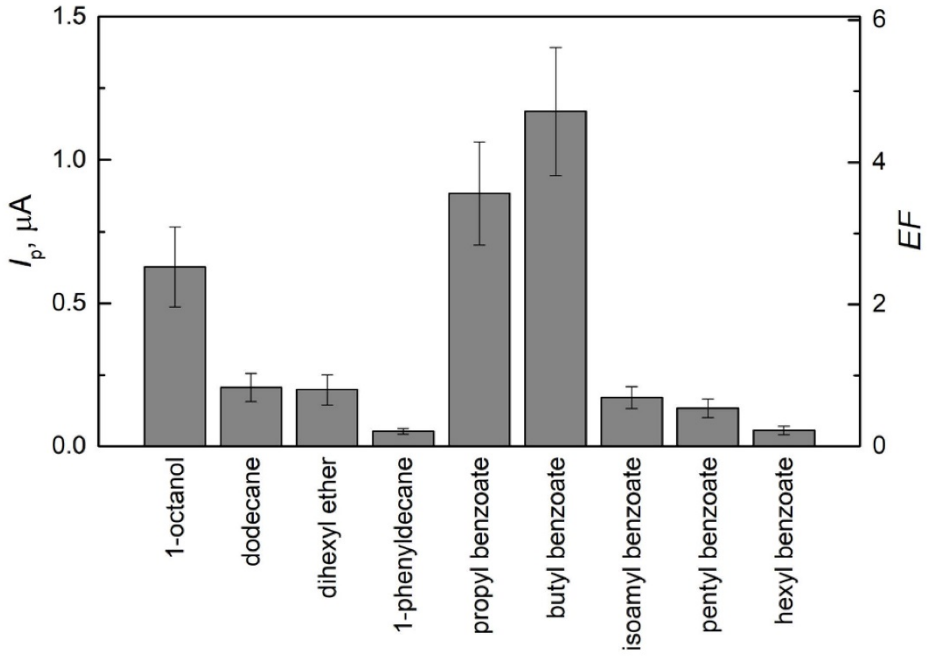
3.2.1 Optimization of supported liquid membrane (SLM) composition

SLM choice proves to be crucial for HF-LPME selectivity, efficiency and repeatability. Optimum liquid for SLM is non-volatile, negligibly miscible with water and with good solubility of the analyte. For polar analyte such as HVA, these properties are apparently contradictory. In our case the SLM choice is also limited to solvents that are electrochemically inactive at the used potential range, pH and electrode material. Cost, environmental impact and toxicity are other factors to be considered. For all these reasons, the SLM solvent has to be found experimentally. Tested solvents included common solvents for HF-LPME extraction[50] of polar analytes: 1-octanol, dihexyl ether, phenyldecane, and dodecane. Selected benzoic acid esters were also included because butyl benzoate was previously successfully used for HF-LPME of VMA structurally related to HVA [40].

HVA concentration of 50 μmol L^{-1} used to mimic elevated HVA levels in urine of infants. Used acceptor and donor solutions were 0.1 mol L^{-1} NaOH and 0.1 mol L^{-1} HCl, respectively. Butyl benzoate SLM was found to be optimum solvent among the tested ones, because it provided the highest HVA peak current (I_p) (Fig. 4). Enrichment factor (EF) was calculated as the ratio of I_p of HVA after HF-LPME and I_p of HVA without extraction at pH corresponding to pH of acceptor phase. Corresponding EF in the case of butyl benzoate SLM was 4.67±0.89. Relative standard deviation (RSD) of I_p and EF, which was used to characterize repeatability, was approximately 18% and comparable for all tested SLMs.

Furthermore, butyl benzoate SLM at the used hollow-fiber is transparent leading to easier handling in comparison with more commonly used translucent 1-octanol SLM.

Fig. 4. Peak currents (I_p) and enrichment factors (EF) of tested SLMs for HVA DPV-determination after HF-LPME. Acceptor solution: 0.1 mol L^{-1} NaOH, V_{AS} 10 µL; Donor solution: 50 µmol L^{-1} of HVA in 0.1 mol L^{-1} HCl, V_{DS} 10 mL; Extraction time (t_{ext}) 30 min; BDDE pre-treatment: E_{act} = −1.0 V, t_{act}= 15 s. The coverage intervals are on the significance level α = 0.05.



3.2.2 Optimization of donor and acceptor solutions

Compositions of acceptor solution and donor solution are critical for efficiency, selectivity, and repeatability of DPV determination of HVA at BDDE after HF-LPME. Maximum extraction efficiency was presumed to be found at low pH of donor solution and at high pH of acceptor solution, with regards to HVA acidic dissociation constants, i.e., pKa1 = 4.35 and pKa2 = 10.34. Concurrently, there is an effect of pH on DPV peak current of HVA in acceptor solution which increases with decreasing pH. Hence, the pH of acceptor solution affects HVA peak height after HP LPME in two contradictory ways. High pH is optimal from the point of view of HF LPME while low pH is optimal from the point of view of final DPV determination. The optimum pH of both acceptor and donor solution for HVA determination

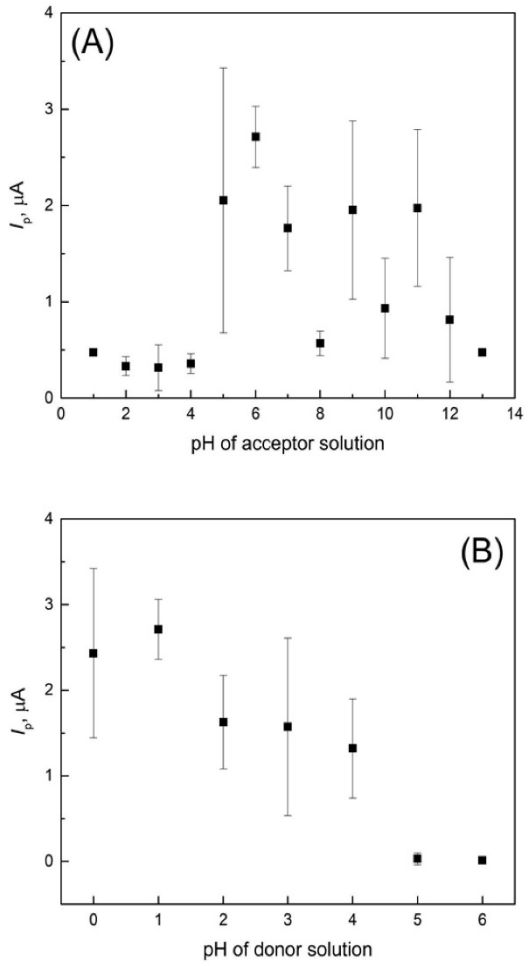
after HF-LPME is thus the question of a suitable compromise and had to be determined experimentally.

The effect of acceptor solution pH on HVA peak current (Fig. 5A) was investigated using 0.1 mol L^{-1} HCl as the donor solution with HVA addition (50 µmol L^{-1}). Acceptor solutions were realized by NaOH (0.1 mol L^{-1}), PBS of pH 2-12 (0.1 mol L^{-1}), and HCl (0.1 mol L^{-1}). PBS ionic strength was adjusted (by NaCl addition) to I = 0.55 mol L^{-1}.

At pH range from 1 to 4 of acceptor solution, HVA was present predominantly in its neutral form in acceptor solution as well as in donor solution. The main extraction process was an equilibration with no effective entrapment or pre-concentration. Thus, only small voltammetric signals were recorded, despite the fact that the acidic pH is generally the optimum for DPV determination of HVA [37, 38]. At pH 5 and higher, entrapment of negatively charged HVA in the acceptor solution has led to increased extraction efficiency and higher recorded peak currents. Highest and sufficiently repeatable HVA peak current was obtained at pH 6 of the tested acceptor solution, which was chosen as optimum. Low repeatability at pH 5 was apparently caused by pH of acceptor phase being close to HVA pK_{a1} = 4.35, along with lower PBS buffering capacity at that pH. Under more basic conditions of pH 7 and higher, the peak currents were decreasing inconsistently, often with poor repeatability, especially from pH 9 to 12.

Analogously, pH of the used donor solution was investigated from pH 0 to 6 (Fig. 5B). For these tests, the of acceptor solution pH was set to its optimum value of 6. Solution of pH 0 was realized by 1 mol L^{-1} HCl. The most repeatable and the highest HVA peak current was recorded using donor solution of pH 1 and, therefore, this pH value was chosen as optimum. At donor solution of pH 5 and higher, only insignificant signal has been recorded, confirming the previously proposed mechanism of HF-LPME resulting in negligible transport of negatively charged form of HVA from donor solution into the SLM.

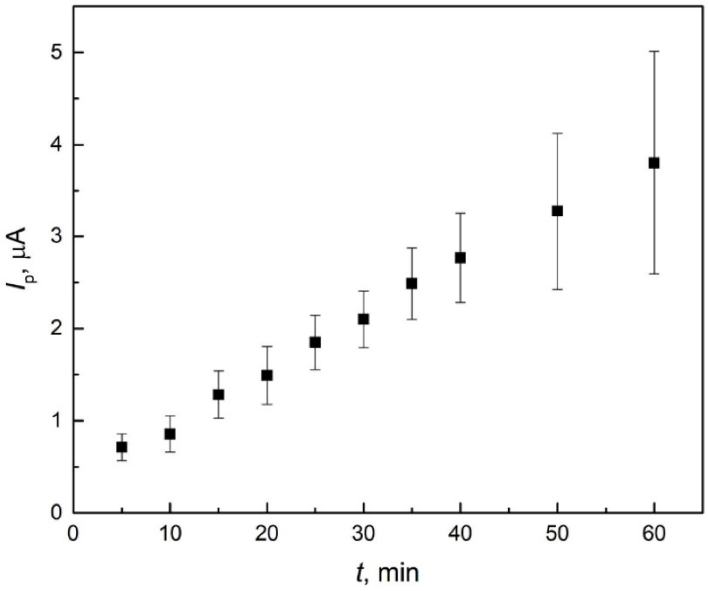
Fig. 5. Dependence of DPV peak current of HVA after HF-LPME on pH of acceptor solution (A) and donor solution (B). (A) V_{DS} 10 mL, 0.1 mol L^{-1} HCl with 50 µmol L^{-1} of HVA; Acceptor solution: pH: 1–13; (B) Acceptor solution: PBS pH 6, V_{AS} 10 µL; Donor solution: pH: 0–6 with HVA (50 µmol L^{-1}). Acceptor and donor solutions were 0.1 mol L^{-1} PBS, 1 mol L^{-1} HCl, 0.1 mol L^{-1} HCl, or 0.1 mol L^{-1} NaOH. Ionic strengths of PBS were constant (I = 0.55 mol L^{-1}). SLM: butyl benzoate, t_{ext} = 30 min; BDDE pre-treatment: E_{act} = −1.0 V, t_{act} = 15 s.



3.2.3 Optimization of extraction time

Fig. 6 shows the dependency of DPV peak current of HVA after HF-LPM on extraction time (t_{ext}) from 5 to 60 min. The registered peak currents increased approximately linearly with increasing t_{ext}. However, application of t_{ext} longer than 40 min lowered the repeatability noticeably (this was probably caused by progressive dissolution of butyl benzoate from the SLM to the stirred donor solution). Finally, t_{ext} 30 min was chosen as optimum due to the best ratio of peak currents to their standard deviation.

Fig. 6. Dependence of DPV peak current of HVA after HF-LPME on extraction time t_{ext}. Donor solution: V_{Don} 10 mL; 0.1 mol L^{-1} HCl with 50 µmol L^{-1} of HVA. Acceptor solution: PBS pH 6; SLM: butyl benzoate, t_{ext} 30 min; BDDE pre-treatment: E_{act} = −1.0 V, t_{act} = 15 s.



3.3 DPV concentration dependence after HF-LPME

Under the optimum conditions, HF-LPME-DPV concentration dependence of HVA was investigated in the range from 0.25 to 100 µmol L^{-1}. In Fig. 7A and Fig. 7B, DP voltammograms are depicted in two consecutive concentration ranges (Fig. 7A from 0.25 to 10 µmol L^{-1}, Fig. 7B from 10 to 100 µmol L^{-1}) corresponding to two linear regions. Figures of merits are summarized in Table 1. *LOQ* amounted to 1.2 µmol L^{-1} and *LOD* to 0.4 µmol L^{-1}.

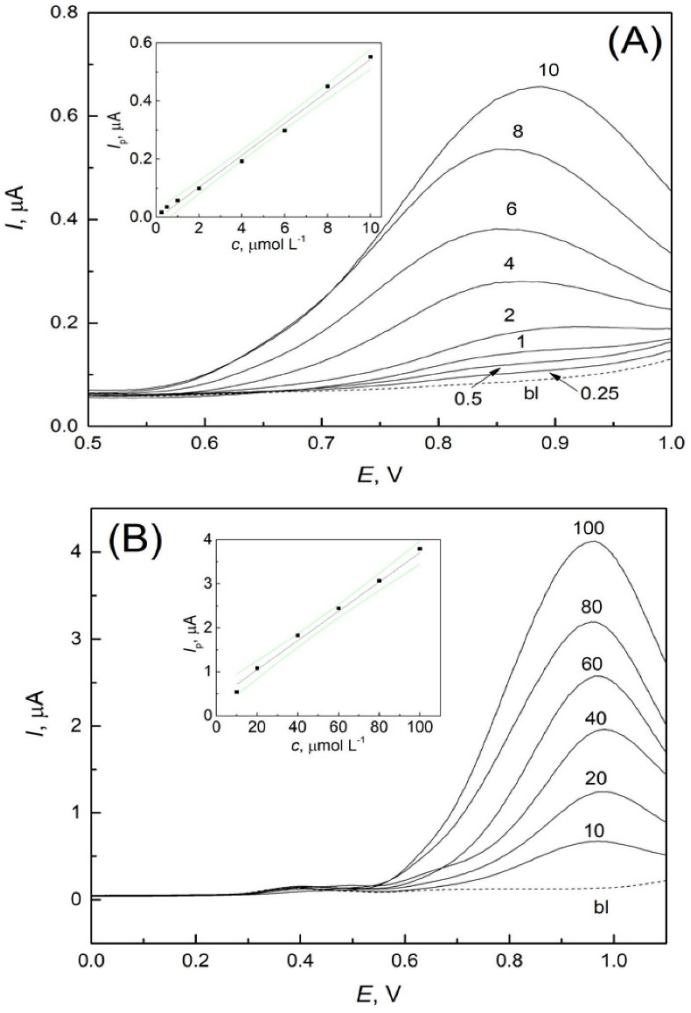
Table 1

Figures of merits of HF-LPME-DPV determination of HVA. The coverage intervals (older used term was "confidence" intervals) were calculated on the significance level $\alpha = 0.05$. Other parameters are same as in Fig. 7.

Concentration range (μmol L^{-1})	**Slope (mA mol^{-1} L)**	**Intercept (μA)**	**Correlation coefficient**	***LOQ*** **(μmol L^{-1})**	***LOD*** **(μmol L^{-1})**
10-100	33.0 ± 1.6	0.380 ± 0.099	0.9952	-	-
0.4-10.0	55.0 ± 2.1	−0.007 ± 0.012*	0.9928	1.2	0.4

* Insignificant on the significance level $\alpha = 0.05$.

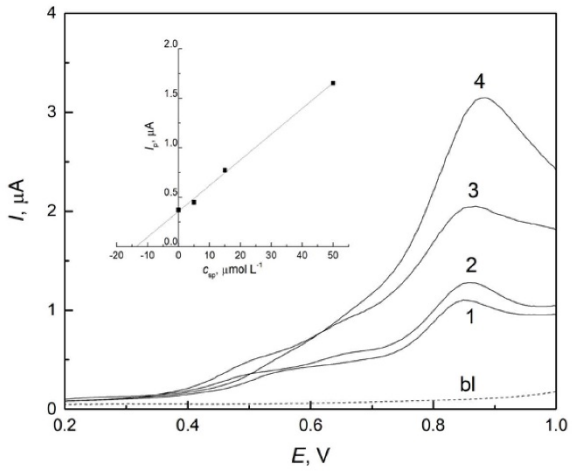
Fig. 7. DP voltammograms of HVA after HF-LPME. Acceptor solution 0.1 mol L^{-1} PBS of pH 6. A) Donor solution 0.1 μmol L^{-1} HCl with added HVA from 0.25 μmol L^{-1} to 10 μmol L^{-1}; (B) Donor solution 0.1 μmol L^{-1} HCl with added HVA from 10 to 100 μmol L^{-1}. The numbers next to the curves correspond to HVA concentrations in μmol·L^{-1}. Dotted curve (bl): blank extraction from donor solution. SLM was prepared using butyl benzoate, t_{ext} 30 min; BDDE pre-treatment: E_{act} = −1.0 V, t_{act}= 15 s. Inset: HF-LPME-DPV concentration dependence of HVA, including coverage intervals (green line).



3.4 DPV determination of HVA in real sample of human urine after HF-LPME

Finally, the verification of the developed method was performed by analysis of a real human urine sample (provided by 29-year-old volunteer, healthy male). To prepare the donor solution, 9 mL of centrifuged non-diluted human urine was mixed with 1 mL of 1 mol L^{-1} HCl, and eventually with addition of HVA standard to the concentrations of 5, 15, and 50 μmol L^{-1}. DP voltammetric curves of urine after HF-LPME are shown in Fig. 8. Contrary to direct voltammetric analysis of HVA in urine, HF-LPME-DPV provided analytically usable and well-developed peak in non-spiked as well as spiked urine samples. It is notable that in the case of urine samples, the background of HF-LPME-DP voltammograms is elevated compared to the deionized water samples. This effect can be explained by presence of electrochemically oxidizable interferences that can be co-extracted with HVA, e.g., uric acid, ascorbic acid, or products of cytochrome P450 clearance pathway [65]. For this reason, standard addition method was applied to mitigate the effects caused by presence of these compounds in matrix. The tested urine sample contained 13.5 ± 1.3 μmol L^{-1}, RSD = 9.3 % (n=5). This method was found to be adequately sensitive and selective to be used as a tool for preliminary screenings of increased levels of HVA.

Fig. 8. DPV determination of HVA in urine samples after HF-LPME. Curves: urine (1); urine with additions of HVA standards to concentration of 5 μmol L^{-1} (2), 15 μmol L^{-1} (3), and 50 (4) μmol L^{-1}. Dotted curve: extraction from donor solution in absence of urine and HVA addition. Donor solution: Centrifuged mixture of 1 mL of 1 mol L^{-1} HCl and 9 ml of urine, without or with addition of the HVA standard. Other conditions are same as in Fig. 7. Inset: plot of standard addition of HVA, concentration values correspond to the real dilution.



4. Conclusions

This study demonstrates the applicability of HF-LPME-DPV for the determination of clinically relevant substances in complicated biological matrices such as urine. Although the HF-LPME of more polar analytes such as HVA provides smaller enrichment factors (*EF*) than for less polar compounds, it can provide an adequate cleanup of the sample. Therefore, the main advantage consists in a significant increase of selectivity, even though the *LOD* and *LOQ* were lowered by the extraction as well. Moreover, the precision of the method can be presumably further improved by automatic, rather than manual manipulation of the process, as it was demonstrated for similar microextraction methods [51]. The newly developed method proved to be applicable for the use in large-scale screenings for elevated HVA concentrations. Finally, due to low consumption of benign reagents and the use of non-toxic BDDE, the method is in good agreement with the principles of "green analytical chemistry".

Competing interests

The authors declare no conflict of interest

Author contributions:

Vojtěch Hrdlička: Formal analysis, Writing - review & editing, Investigation, Writing - original draft, Investigation; ***Jiří Barek:*** Supervision. ***Tomáš Navrátil:*** Supervision, Project administration.

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Supporting information

Differential pulse voltammetric determination of homovanillic acid as a tumor biomarker in human urine after hollow fiber-based liquid-phase microextraction

Vojtěch Hrdlička, Jiří Barek, Tomáš Navrátil

Elimination voltammetry with linear scan (EVLS) [1-6] was used for elucidation of the electrode mechanism [7, 8]. According to the theory of EVLS, total recorded current (I) can be expressed as a sum of partial currents (I_j) (Eq. S1): kinetic current (I_k), charging current (I_c), diffusion current (I_d) and the irreversible current (I_{ir}) [4].

$$I = \Sigma I_j = I_k + I_c + I_d + I_{ir} \quad \text{(S1)}$$

Concurrently, it is assumed that each partial current I_j can be expressed as a function of scan rate and a function of potential in the following form (Eq. S2) [4].

$$I_j = W_j(v)\, Y_j(E) = v^x Y_j(E) \quad \text{(S2)}$$

I_j represents selected partial current, $W_j(v)$ represents a function of the scan rate, $Y_j(E)$ is a function of the potential, and v is the scan rate. EVLS was calculated from a few sets of cyclic voltammograms of 100 μmol L^{-1} HVA in 0.1 mol L^{-1} with the scan rates from 0.020 V s^{-1} – 0.160 V s^{-1} (Fig. S1) and 0.160 V s^{-1} – 1.280 V s^{-1} (Fig. S2) at cathodically pretreated BDDE (t_{acc} = 15 s, E_{acc} = −1 000 V). As can be seen from Fig. S1 and FigS2, respectively, after application of elimination equations [1-6], only diffusion current remains significant. On the other hand, the other contributions (kinetic, capacitance, and irreversible currents) could be almost eliminated and proved to be negligible. Therefore, it can be concluded that, HVA electrode process is mainly controlled by diffusion in both experimental sets.

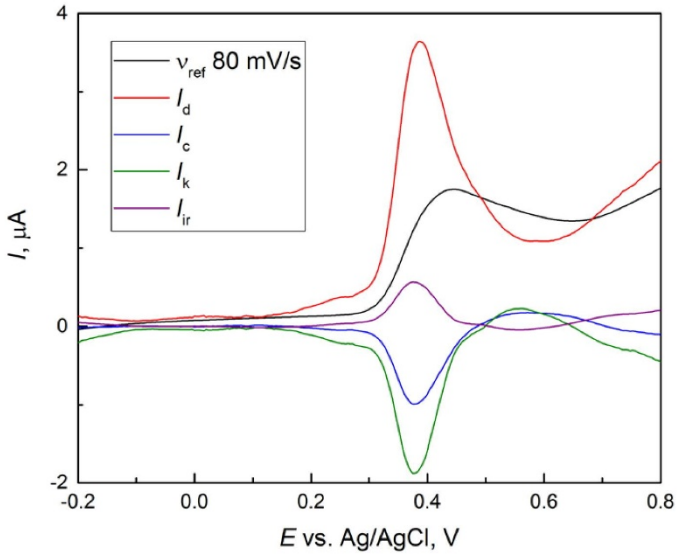

Fig S1. EVLS recordings of 100 µmol L^{-1} HVA in 0.1 mol L^{-1} NaOH with the scan rates from 0.020 V s^{-1} to 0.160 V s^{-1}, ν_{ref} = 0.080 V s^{-1}, cathodically pretreated BDDE (t_{acc} = 15 s, E_{acc} = −1.0 V).

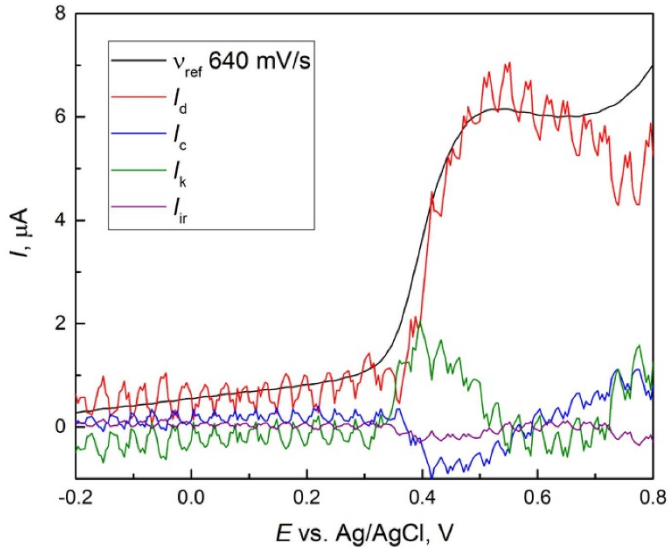

Fig S2. EVLS recordings of 100 µmol L^{-1} HVA in 0.1 mol L^{-1} NaOH with the scan rates from 0.160 V s^{-1} – 1.280 V s^{-1}, ν_{ref} = 0.640 V s^{-1} and at cathodically pretreated BDDE (t_{acc} = 15 s, E_{acc} = −1.0 V).

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Confirmation of participation

1. **Vojtěch Hrdlička**, Tomáš Navrátil, Jiří Barek: Application of hollow fibre based microextraction for voltammetric determination of vanillylmandelic acid in human urine. Journal of Electroanalytical Chemistry 835, 130-136.
   5-year impact factor: **3.519**,
   Percentage of participation of Mgr. Hrdlička ~ **85 %**.

2. **Vojtěch Hrdlička**, Marta Choińska, Beatriz Ruiz Redondo, Jiří Barek, Tomáš Navrátil: Determination of heavy metal poisoning antidote 2, 3-dimercapto-1-propanesulfonic acid using silver solid amalgam electrode. Electrochimica Acta 354, 136623.
   5-year impact factor: **5.478**,
   Percentage of participation of Mgr. Hrdlička ~ **40** %.

3. **Vojtěch Hrdlička**, Jiří Barek, Tomáš Navrátil: Differential pulse voltammetric determination of homovanillic acid as a tumor biomarker in human urine after hollow fiber-based liquid-phase microextraction. manuscript submitted.
   Percentage of participation of Mgr. Hrdlička ~ **85** %.

I declare that the percentage of participation of Mgr. Vojtěch Hrdlička at the above given papers corresponds to above given numbers.

Prague 10th August 2020

prof. RNDr. Jiří Barek CSc.

List of publications, oral and poster presentations

Impacted journal articles

1. Nováková K, **Hrdlička V**, Navrátil T, Vyskočil V, Barek J: Determination of 5-nitroindazole using silver solid amalgam electrode. Monatshefte für Chemie-Chemical Monthly 146 (5), 761-769.

2. Nováková K, **Hrdlička V**, Navrátil T, Harvila M, Zima J, Barek J: Application of silver solid amalgam electrode for determination of formamidine amitraz. Monatshefte für Chemie-Chemical Monthly 147 (1), 181-189.

3. **Hrdlička V**, Navrátil T, Barek J, Ludvík J: Electrochemical behavior of polycrystalline gold electrode modified by thiolated calix [4] arene and undecanethiol. Journal of Electroanalytical Chemistry 821, 60-66.

4. **Hrdlička V**, Navrátil T, Barek J: Application of hollow fibre based microextraction for voltammetric determination of vanillylmandelic acid in human urine. Journal of Electroanalytical Chemistry 835, 130-136.

5. **Hrdlička V**, Choińska M, Ruiz Redondo B, Barek J, Navrátil T: Determination of heavy metal poisoning antidote 2, 3-dimercapto-1-propanesulfonic acid using silver solid amalgam electrode. Electrochimica Acta, 136623.

6. **Hrdlička V**, Barek J, Navrátil T: Differential pulse voltammetric determination of homovanillic acid as a tumor biomarker in human urine after hollow fiber-based liquid-phase microextraction. submitted.

Oral presentations

1. **Hrdlička V**, Navrátil T, Šestáková I, Barek J, Ludvík J: The Use of Self-Assembled Monolayer of Thiolated Calix[4]arene on Polycrystaline Gold Electrode Surface. XXXV. Modern Electrochemical Methods, Jetřichovice u Děčína, Czech Republic (18. - 22. 5. 2015).

2. **Hrdlička V**, Navrátil T: Hollow Fibre Microextraction and Voltammetric Detection of Homovanillic and Vanillylmandelic Acid. Seminar of Students of JHI 2016 – Liblice, Czech Republic (10. - 11. 5. 2016).

3. **Hrdlička V**, Barek J, Navrátil T: Possibilities of Hollow Fiber Microextraction and Voltammetric Detection of Homovanillic acid. XXXVI. Modern Electrochemical Methods, Jetřichovice u Děčína, Czech Republic (23. - 27. 5. 2016).

4. **Hrdlička V**, Barek J, Navrátil T: Voltammetric determination of homovanillic acid at boron doped diamond electrode for hollow fibre microextraction. 12th International Students Conference Modern Analytical Chemistry, Prague, Czech Republic (22. - 23. 9. 2016).

5. **Hrdlička V**, Navrátil T: Hollow Fibre Liquid-Phase Microextraction of Catecholamine Based Biomarkers. Seminar of Students of JHI 2017 – Liblice, Czech Republic (9. - 10. 5. 2017).

6. **Hrdlička V**, Barek J, Navrátil T: Hollow-fibre Based Microextraction and Voltammetric Determination of Vanillylmandelic Acid. XXXVII International Conference on Modern Electrochemical Methods, Jetřichovice, Czech Republic (15 - 19. 5. 2017).

7. **Hrdlička V**, Barek J, Navrátil T: Miniaturized Boron Doped Diamond Film Electrode for Neuroblastoma Biomarkers Determination. 13th International Students Conference on Modern Analytical Chemistry Location: Prague, Czech Republic (21. - 22. 9. 2017).

8. **Hrdlička V**, Barek J, Navrátil T: Supported Liquid Membranes for Hollow-Fibre Based Microextraction and Voltammetric Determination of Neuroblastoma Biomarkers. XXXVIII International Conference on Modern Electrochemical Methods, Jetřichovice, Czech Republic (21. - 25. 5. 2018).

9. **Hrdlička V**, Navrátil T: Hollow Fibre Microextraction and Voltammetric Detection of Homovanillic and Vanillylmandelic Acid. Seminar of Students of JHI 2018, Czech Republic (12. - 13. 6. 2018). **Excellent speech award**

Poster presentations

1. **Hrdlička V**, Barek J, Navrátil T: Voltammetric detection of homovanillic and vanillylmandelic acid for hollow fibre microextraction. EuCheMS Chemistry Congress, Seville, Spain (11. - 15. 9. 2016).

2. **Hrdlička V**, Barek J, Navrátil T: Hollow fiber microextraction and voltammetric detection of Vanillylmandelic Acid. 5th International Conference and Expo on Separation Techniques, Paris, France (23. – 25. 10. 2017).

3. **Hrdlička V**, Barek J, Navrátil T: Hollow fiber microextraction and voltammetric detection of Vanillylmandelic Acid. 17th ESEAC International Conference on Electroanalysis, Rhodes, Greece (3. – 7. 6. 2018).

4. **Hrdlička V**, Ruiz Redondo B, Choinska M, Navrátil T, Schwarzová K: Voltammetric determination of heavy metal poisoning antidote DMPS on amalgam electrodes. 11th Central European Meeting on Molecular Electrochemistry CEMME, Chemnitz, Germany (17 - 19. 11. 2019).

Internship

1. Erasmus+ internship at Electrochemistry and Corrosion Group of Prof. Christopher Brett, University of Coimbra, Faculty of Science and Technology, Department of Chemistry, Portugal (1. 4. 2019 - 30. 6. 2019).

Grants

1. Principal investigator: Electromembrane separation and voltammetric determination of biomarkers in urine. Grant Agency of Charles University, project GAUK630216.

2. Team member: Electrochemically active calix[4]arenes and their use for preparation of new receptors and (bio)sensors. Grant Agency of Czech Republic, project GA CR 13 - 21704S.

3. Team member: New strategies for improving sensing properties of novel electrode materials via their surface pretreatment or modification. Grant Agency of Czech Republic, project GA CR 20-01589S.

4. Team member: Electrochemical and analytical aspects of the transport of addictive and psychotropic drugs across the model biological barriers. Grant Agency of Czech Republic, project GA ČR 20-07350S.